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BACTERIOLOGY

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BACTERIOLOGY

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To the Memory of

JOSEPHINE HALL BUCHANAN

This Book Is Affectionately Dedicated

PREFACE

Advances in the field of microbiology (or bacteriology) have been very great in recent years, increments to our knowledge have come in geometric progression. Revision of a basic text in the subject is therefore increasingly necessary, but it is also increasingly difficult. The area to be covered has greatly broadened, the subject has become more complex, and its importance is every day more evident. The rapid advances in physics, genetics, biology, taxonomy, immunology, physiology, biochemistry and in the applications of microbiology to every day life, to agriculture, home economics, medicine and public hygiene have all combined to make adequate and concise presentation of the elements of the science troublesome.

That there is need for a text covering the general field of bacteriology without special emphasis upon the medical aspects of the science is evident from the wide use and acceptance of previous editions. For the most part, the text has been rewritten, but without material change in the order of presentation and arrangement. Particular emphasis has been given to the modern developments in cellular morphology and physiology and to the many technologies in which microorganisms are significant.

In a somewhat larger number of cases the attempt has been made to give the root meanings and the derivations of some of the technical terms that are used in bacteriology. In many cases the meaning of technical and scientific names may be clarified by giving the classical origin. This is simple when the word is derived from the Latin, but problems of transliteration arise if the word comes from the Greek. For example, the name of the disease psittacosis comes from the Greek ψιττακός, meaning a parrot. Strict transliteration would give psittakós, but the better method is to follow the system used by the Latins and render it *psittacus*.

In general the nomenclature of the bacteria and the viruses used in the sixth edition of Bergey's *Manual of Determinative Bacteriology* has been employed. Descriptions of the more important groups are given in the body of the text, and keys to most of the genera included in the appendices. The numbers of molds included in Appendix C has been increased, but some space has been conserved by placing twelve illustrations on each page.

I am sincerely grateful to Miss Ruth Knuths, to Mrs. Lucy Wendel and to Mrs. J. C. Cunningham for their invaluable assistance in the preparation of the manuscript and to Mr. John Staby for much help in the preparation of illustrations.

Ames, Iowa R. E. BUCHANAN

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CHAPTER 1

The Beginnings of Bacteriology

Bacteriology has chiefly to do with the groups of microscopic plants called the *bacteria*. Not long after the discovery of these minute organisms they were found to have considerable practical significance; some produced disease and some produced fermentations, some were beneficent and some were harmful. In consequence more and more attention was paid to their study, and many special methods for their observation were developed. These methods promptly found application also in the study of other microscopic forms of life, including such forms as the *molds* and *yeasts* grouped with plants, certain *protozoa* usually included in the animal kingdom, and more recently those ultramicroscopic forms termed the *viruses*. The fact that all these forms came to be studied in the bacteriological laboratory led some observers to expand the meaning and scope of the term bacteriology to include consideration of all the microorganisms observed by the special methods which had been developed in the first instance for the study of bacteria. Other students preferred to use the somewhat more inclusive and appropriate term, *microbiology,* defined as the science that has to do with all microscopic forms of life. Both terms, bacteriology and microbiology, may therefore be defined to include consideration of the *bacteria, yeasts, molds, viruses,* and certain *protozoa*.

WHY BACTERIOLOGY

Bacteriology is included with increasing frequency as an integral part of many college curricula. There are two principal reasons for such inclusion. First, bacteriology has come to be recognized as a discipline which contributes much to the useful background of information of the educated man, enabling him more successfully to fit himself to the environment in which he lives. Second, a knowledge of bacteriology is an essential part of the training for many vocations; for some it is an essential prerequisite.

Bacteria and other microorganisms have many definite relationships to the maintenance of health in man, animals and plants. A knowledge of these is helpful to all of us in understanding the problems of personal hygiene and of protection from disease; such knowledge is excellent insurance against medical quackery and fraud. The community is intimately concerned with the application of bacteriological principles to prevention of disease transmission. Intelligent cooperation with health officers and sanitarians is furthered by some bacteriological knowledge on the part of the citizen.

Bacteria have many relationships to man other than those concerned with disease and health. Knowledge of these helps us to understand the many changes for which they are responsible in nature, such as the phenomena of decay, putrefaction, and fermentation which enter frequently into the experiences of everyday life. Ability to read and understand the current literature of popular science, and even the daily press, is dependent upon some acquaintanceship with bacteria as they relate to modern living.

Adequate training for many of the specific vocations involves acquisition of special knowledge of bacteriology and its applications. The content of the science of bacteriology has grown so rapidly in recent years, and its vocational utilizations have been so diverse, that several important subdivisions of the subject have developed with numerous highly specialized applications. For example, the discoveries of bacteriologists during the past fifty years have quite revolutionized human and veterinary medicine. It is essential that the medical and the veterinary student master the elements not only of general or fundamental, but *medical bacteriology* as well. He should know the factors which determine the resistance of the body to disease-producing bacteria and other microorganisms, he should be conversant with *immunology*. Some knowledge of these subjects is important not only to the physician, but also to all concerned directly or indirectly with the prevention and cure of disease. The sanitarian, sanitary engineer, and public health official make practical use of information supplied by *sanitary bacteriology;* they must needs concern themselves particularly with the ways in which pathogenic (disease-producing) bacteria are disseminated and the means by which their distribution may be prevented. The problems of water purification and of sewage disposal are problems of elimination of bacteria on the one hand or of their destruction on the other.

Living cells, no matter what their origin, whether plant, bacteria, animal, or man, have many fundamental likenesses. The ease with which we may grow the cells of microorganisms and study them in the laboratory has made them most useful in getting at basic problems in physiology. The facts discovered in *physiological bacteriology* have found wide application in understanding the physiology of other living forms. The fact that antagonisms between different kinds of microorganisms exist, that one produces substances definitely harmful to another, has led to the isolation of those substances and to their utilization in the cure of disease. The discovery that there are substances, such as penicillin, which have high therapeutic (curative) value has led to the development of a branch that may be termed *pharmaceutical bacteriology*.

Those who are employed in industry may be vitally concerned with the chemical changes produced by bacteria. Some of these changes are classified as fermentations; the branch of bacteriology which treats of these is *zymology* or *zymotechnique*. Bacteria, yeasts, and molds have many diverse growth requirements. Their rates of growth may in some cases be utilized in determining the concentration of definite chemical compounds. They have become therefore tools for use in making chemical analyses, particularly in determining amounts of hormones, vitamins and similar substances active in minute quantities. There is rapidly developing an *analytical bacteriology* which serves analytical chemistry. Applications of bacteriology to agriculture have been numerous and important. *Soil bacteriology* is concerned with the many changes in soils produced by the activity of bacteria, modifications requisite to soil fertility and to the growth of crops. The changes resulting from the growth of bacteria in milk and its products constitute the subject matter of *dairy bacteriology*. In *food bacteriology* there is emphasis upon the relationships of organisms to the manufacture, deterioration, and preservation of foods, an essential part of training for food technologists. The list of vocational applications might be indefinitely extended.

ORIGINS OF THE SCIENCE OF BACTERIOLOGY

As a science, bacteriology is not as old as the other biological sciences such as botany and zoology. Most of our knowledge of the subject has been developed since 1900, practically all of it since 1860. The small size of bacteria made their study difficult. It was not until nearly the middle of the nineteenth century that bacteria could be observed satisfactorily. Ability to see bacteria and the realization of their great economic importance developed together. Four among the more important factors determining the growth of the science were: (1) the development of microscopes which have increasingly made possible more adequate observation of such minute cells as the bacteria; (2) the controversy which was waged over the theory of spontaneous generation; (3) the development and acceptance of the germ theory of fermentation and decay; and (4) the development and proof that certain bacteria and other microorganisms are capable of producing disease in man, animals, and plants. How have these worked together to develop the modern science of bacteriology?

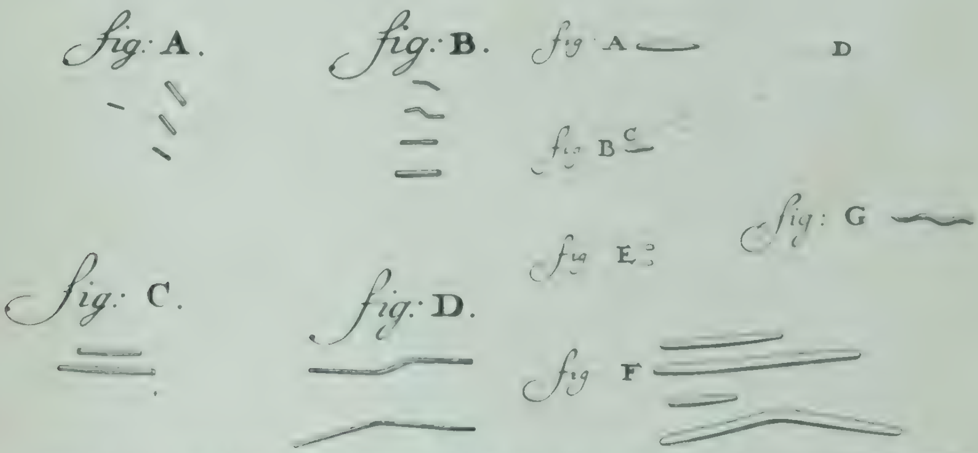

Fig. 1–1. The first illustrations of bacteria prepared by Leeuwenhoek, published in the *Proceedings of the Royal Society of London*. Note that he drew spherical, rod-shaped and spiral cells and that by means of a dotted line indicated that he observed motility on the part of certain individuals. These bacteria were observed in the tartar from teeth and in infusions of decaying material.

How the Development of the Microscope Influenced Bacteriology. Some of the ancient philosophers of Greece suggested that there might be living objects too small to be seen by the eye and that they, through their multiplication in the bodies of men, might be responsible for disease. No means of testing the validity of such a conjecture was known, though it was suggested that some living germs might be the contagion that passed from person to person in an epidemic. Some of the theories of disease causation were indeed not unlike our modern concept of contagious disease production. But laboratory or other controlled experiments were unknown and little or nothing came of the conjectures. Many centuries passed before mechanical aid in the form of the microscope was sufficiently perfected to permit observation and study of such organisms.

Among the earliest and best recorded observations on minute forms of life were those of Leeuwenhoek, a native of Delft in Holland. He became greatly interested in the making of lenses and mounting them ingeniously as simple microscopes. He examined many materials and sent long descriptions of them to the Royal Philosophical Society of London of which he was made a member. Frequently his letters were accompanied by sketches and drawings. Many were published in the Proceedings of the Society. In one of these letters he described living "animalcules" as he termed them which he had observed in the tartar from his teeth. He noted that some of them could swim. His drawings were sufficiently accurate so that one familiar with the microscopic appearance of the bacteria of the mouth may make reasonably accurate conjectures as to the kinds of bacteria he illustrated. In addition to bacteria he observed many protozoa and algae. In Fig. 1–2 may be seen a drawing of one of Leeuwenhoek's simple microscopes with the devices he used for holding the material to be examined, and in focusing accurately.

Leeuwenhoek made few suggestions as to names or classification of the organisms which he saw.

One of the secretaries of the Royal Philosophical Society of London to whom Leeuwenhoek addressed his letters was the British scientist Robert Hooke. He likewise was much interested in lenses and developed a compound microscope with which he studied many minute objects. In 1665 he published a large book called *Micrographia* in which he described many of his observations, among them certain molds, which he figured. (See Fig. 1–3.)

The fact that his microscopes were somewhat unsatisfactory is evident from a statement in his *Observation XXI. Of Moss and several other small vegetative substances* in which he says (p. 135)

> Nay, I have observ'd, that putting fair Water (whether Rain-water or Pump-water, or *May-dew*, or Snow-water, it was almost all one) I have often observ'd, I say, that this Water would, with a little standing, tarnish and cover all about the sides of the Glass that lay under water, with a lovely green; but though I have often endeavour'd to discover with my *Microscope* whether this green were like Moss, or long striped Sea-weed,

or any other peculiar form, yet so ill and imperfect are our *Microscopes*, that I could not certainly discriminate any.

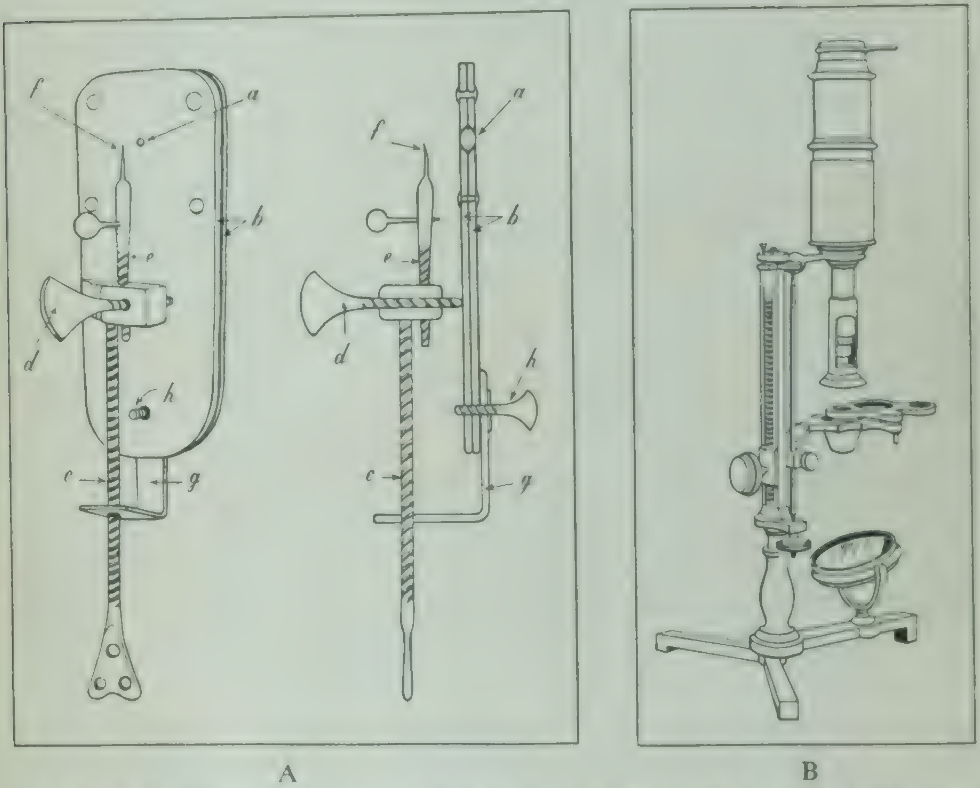

Fig. 1–2. Drawings of two early microscopes. A. Drawing of one of the simple microscopes made and used by Leeuwenhoek. To the left is given a front view, to the right a longitudinal section. Essentially the microscope consisted of a simple glass lens *a* mounted between two brass plates *b*, so fitted that a small part of the glass lens was visible. The object to be examined was mounted on the end of the needle at *f*. The object could be brought into focus and viewed from all sides by use of the several screw adjustments. The screw *c* permitted the object to be raised and lowered, the screw *d* regulated the distance of the object from the lens, the screw *h* facilitated lateral motion. By the use of this relatively simple instrument and the fitting of lenses having high curvature, Leeuwenhoek was able to get a glimpse of many minute microorganisms. B. Drawing of the "Martin" microscope of 1770. A comparison of this microscope with modern compound microscope will show that it has most of the essentials, including the eye piece, the ocular, and the mirror, with arrangements for focusing.

Other students began to use microscopes and to report their observations. One of these, Joblot, a Frenchman, in 1754 wrote an extensive treatise on many microscopic objects. The lack of satisfactory microscopes led to bizarre statements. For example, Joblot figured an organism which he (see Fig. 1–3) described as follows:

"The whole top of its body is covered by a fine well formed mask of a human face, perfectly well made as one may judge from the figure, in which one sees six legs and one tail, emerging from below this mask, which is crowned by a peculiar head dress." Such a description could only have been written as a result of poor microscopic definition and a vivid imagination.

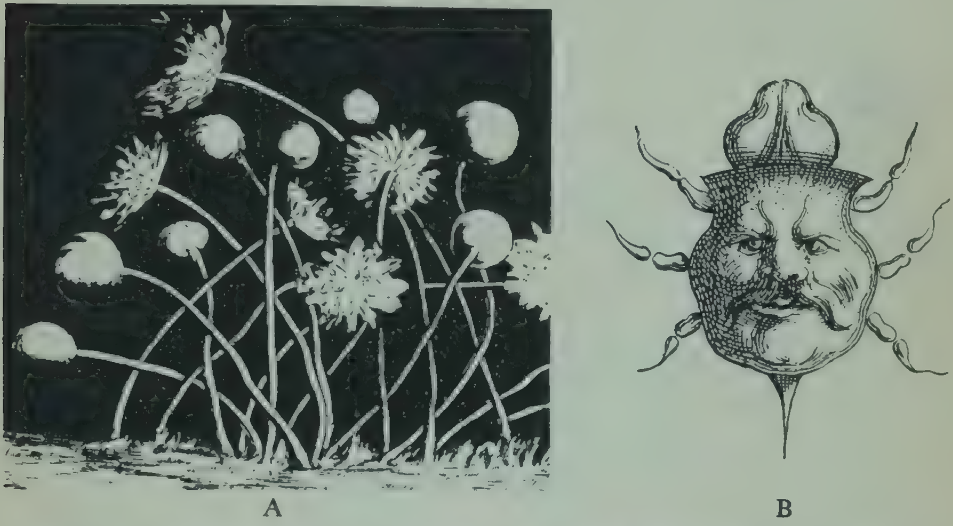

Fig. 1–3. Reproductions of illustrations of living things as seen under the microscope by early observers. A. Robert Hooke (1665) examined a mold, apparently the sporangiophores and spore cases of a *Mucor*. Some of the sporangia are shown as spherical and intact, others are broken open, and the tattered sporangial walls are evident. B. Almost a century later the microscope used by the French scientist Joblot was still unsatisfactory: he published this illustration of what he saw. It seems probable that he observed some tiny mite, and his imperfect lenses led to his belief that the organism had a face or a mask upon its back. Evidently the microscopes of his time were not adequate for accurate observation.

Nearly a hundred years passed before notable further advance was made in observations of minute forms of life as small as the bacteria. During this period the compound microscope was developed to a point that enabled the Danish scientist Müller, in 1773, to examine tiny forms of life more satisfactorily and to publish illustrations and a detailed classification with scientific names. He, like Leeuwenhoek, regarded practically all the forms he studied as animals. From his descriptions and illustrations we know that some were bacteria, some were algae and most were protozoa. He introduced for the first time certain words which we now use as generic names in bacteriology, such as *Bacillus, Vibrio,* and *Spirillum.*

During the next half century still better microscopes were designed and constructed. By their use the German investigator Ehrenberg (1838) made a remarkable survey of microscopic forms of life and published the results in two large volumes. He, too, regarded the organisms which he described as animalcules, but several species of bacteria were included. In fact, it was Ehrenberg who first proposed the name *Bacterium* to designate a genus. Some of the bacteria which he pictured and described are reproduced in Fig. 1–4.

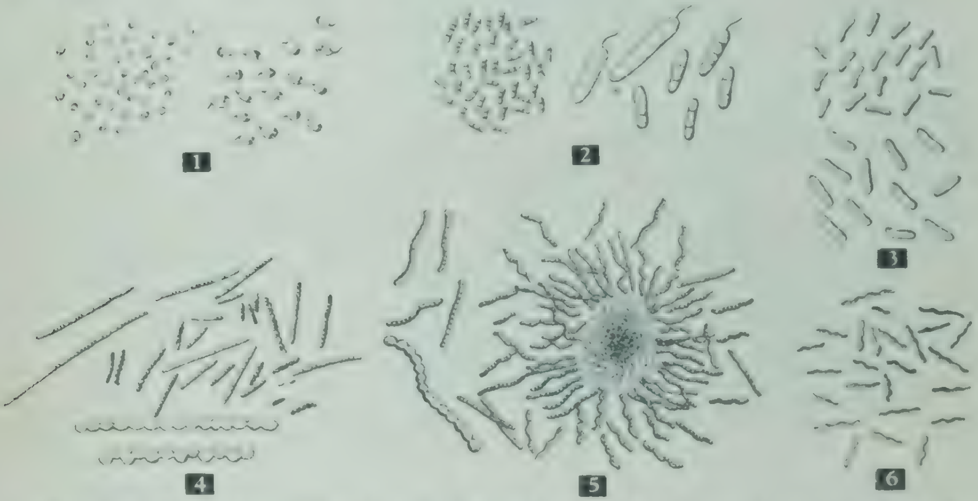

Fig. 1–4. Reproductions of some of the figures of bacteria (regarded as protozoa) published by Ehrenberg in 1838. Following are the names that he gave to these organisms. 1. *Monas termo*. 2. *Bacterium triloculare*. This was the first organism to which the name *Bacterium* was applied. Note that most of the cells seem to have two or three cross walls. Further, many of the cells are shown with a single whip or flagellum at one end. Much confusion in bacteriology has arisen from the fact that the organism described by Ehrenberg has not been found and identified in recent years, and bacteriologists are somewhat uncertain as to what Ehrenberg really saw. 3. *Bacterium punctum* and *Bacterium enchelys*. 4. *Vibrio lineola*. 5. *Vibrio rugula*. 6. *Spirillum tenue*.

Improvements in the microscope still continued. In 1844 Dolland demonstrated the usefulness of the immersion lens, that is, a high-powered objective which was immersed at its tip in water, glycerol, oil, or some clear liquid having a much higher refractive index than air. This permitted the observer to see small objects much more clearly. From this finding were developed the oil immersion objectives so universally used nowadays in bacteriological laboratories. By 1870 Abbé had developed the substage condenser for the microscope. This made possible much better lighting of microscopic objects and the use of lenses which gave still higher magnifications.

Every increase in definition and magnification added to the ability of students to get a better knowledge of the world of microscopic plants and animals. About the middle of the nineteenth century the differentiation of bacteria as one group of microscopic plants, from the protozoa as microscopic animals, was proposed and soon gained acceptance.

Use of better microscopes of his day made it possible for the German botanist Cohn with his pupils to publish a series of papers (1872–1876) which are often regarded as the beginnings of the modern science of bacteriology. He named many kinds of bacteria and developed the first usable classification. He discovered that some bacteria produced resistant cells or spores. Reproductions of some of the illustrations which he drew are shown in Fig. 1–5.

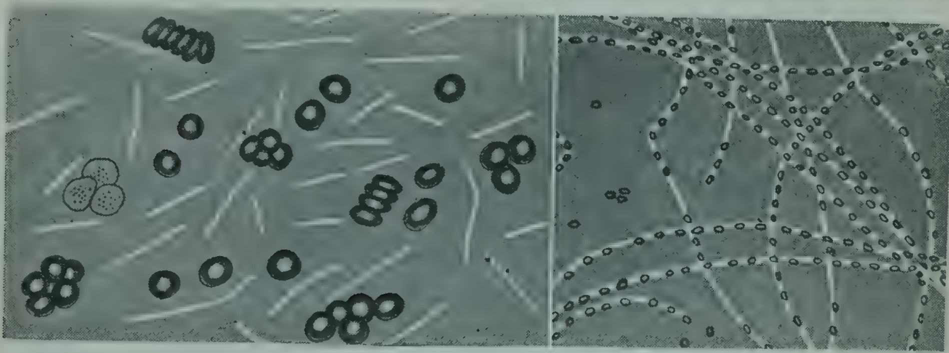

Fig. 1–5. Illustrations of the organism which causes the disease anthrax, published in Cohn's *Beiträge* (1876) by Koch. A. Red corpuscles, white blood cells and the anthrax bacillus (*Bacillus anthracis*) as found in the blood of cattle. B Chains of the anthrax bacillus showing spores.

The microscopes which were available to Cohn gave magnifications practically equal to those of the laboratory microscopes of today. The reasons why the physicist has been unable to produce more powerful microscopes for direct vision are comparatively simple. He can conquer the problem of producing lenses that should yield higher magnifications, but they do not prove satisfactory when put into use. Their failure is due to the very nature of light itself. One of the laws of optics demonstrated by the physicist is to the effect that a clear view cannot be secured when the object which is being examined is smaller than one-half the wavelength of the rays of light which are being used for illumination. The microscopes usually used in the bacteriological laboratory have sufficiently great magnifying power to make visible objects as small as can be seen by the use of visible light.

An increase in magnification does increase the apparent size of the object under examination, but the outlines and details become less distinct, they are blurred.

The resolution of a microscope is defined in terms of the distance which two objects must be separated in order to be seen as two distinct objects in the image. The microscopist has found that this distance is always directly proportional to the wavelength of the light (or other radiation) used for its observation, and inversely proportional to the numerical aperture of the lens. The mathematical formulation is

$$d = \frac{0.5\lambda}{n}$$

in which d = shortest distance by which two objects may be separated and still give two distinct images.

λ = wavelength of light (or other radiation used)

n = numerical aperture of lens used.[1]

Inasmuch as violet light has a shorter wavelength than red light, a microscope gives better resolution when violet light is used for observation. This means that with a good microscope and violet light objects can be observed as separated if they are a little more than a tenth of a micron (about 120 millimicrons) apart.

The use of light or radiation having wavelengths shorter than those of the violet for observation encounters two difficulties. The usual optical glass is quite opaque to these rays, and ultraviolet light cannot be used for vision. In the ultraviolet range of the spectrum quartz lenses may be substituted for glass, and the fluorescent[2] screen or the photographic plate substituted for the eye. By this means it has been found possible to photograph objects a little less than a tenth of a micron in diameter (75 $m\mu$).

For the observation of the general or gross structure of bacteria the usual light microscope is an efficient instrument. When it is remembered that the parts of the bacterial cell that one may wish to observe are small fractions of a micron in thickness and that certain microorganisms such as many rickettsiae and viruses are still smaller, it is evident that the light microscope cannot give resolution to permit of sufficient magnification for adequate study.

---

[1] The numerical aperture of a lens is determined by the curvature of the lens and by the refractive index of the medium through which the light passes from the focal point to the lens. It is defined as the product of this refractive index by the sine of one half the angle of the cone. If r = refractive index of the medium, and θ = the angle of the light cone, then $n = r \sin \theta$

[2] A fluorescent material is one which absorbs light of one wavelength (such as ultraviolet) and emits light of a longer wavelength, in this case a wavelength in the visible spectrum.

This is not at all the same thing as saying that the "magnifying power" of a microscope may not be increased. It can be. But no advantage accrues through increased magnification beyond a certain point if there is not increased resolution as well.

The development of the modern electron microscope has made possible greater resolution and magnification so that by use of the photographic plate or the fluorescent screen many details may be observed which could not be seen otherwise.

The resolving power of the human eye unaided by lenses is about 0.2 mm. ($200\ \mu = 200{,}000\ m\mu$), that of a good electron microscope may be $4\ m\mu$, or a useful magnification of about 50,000 diameters.

Fig. 1–6. Compound binocular microscope of the type commonly used in the bacteriological laboratory.

The electron microscope uses as a source of radiation a thermionic cathode from which electrons are emitted at very high speeds. Lenses of glass or other materials are not used, the necessary bending of the path of the electrons (the focusing of the beam) is accomplished by means of a series of magnetic fields. Magnets replace the lenses of the light microscope, since they diffract the beams of electrons in much the same manner as lenses diffract the rays of light. In a light microscope there is usually a substage condenser that focuses light upon the object to be examined, similarly the first magnetic field determines the irradiation of the specimen under examination. The beam after passing through the object under observation then traverses a second magnetic field corresponding to the objective of a light microscope, and a part of it also through a third field corresponding

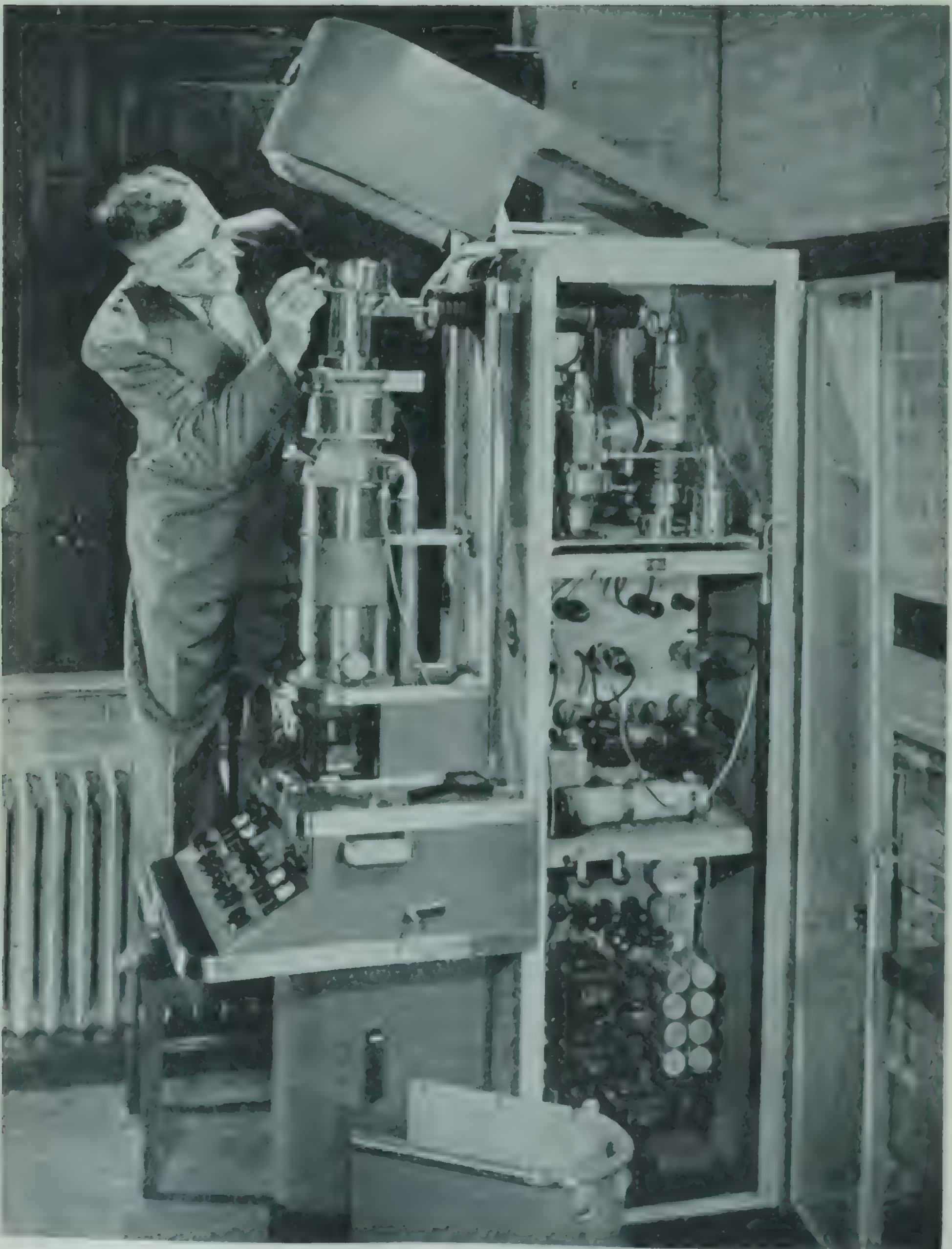

Fig. 1–7. Electron microscope, open to show the microscope itself and the operating parts. This instrument enables the bacteriologist to observe objects much smaller than can be seen with the light microscope. (Courtesy of Department of Physics, Iowa State College.)

to the ocular, which focuses it upon the photographic plate or the fluorescent screen.

The great advantage of the electron microscope is its high resolution. But it has disadvantages. Objects to be examined must be dried and examined in a high vacuum. In general living objects cannot be studied. No colors can be observed.

The Influence of the Controversy over the Theory of Spontaneous Generation on the Early Development of Bacteriology. Some of the old Greek philosophers discussed at some length the origin of living things, particularly the possibility of certain animals or plants developing without parents, arising *de novo*. Some of them surmised, for example, inasmuch as very small frogs were not to be found in nature, that frogs originated spontaneously from the mud of ponds and streams. They concluded that vermin was produced by some kind of a fermentation or other similar process in organic matter. However, it did not require much study to find that frogs laid eggs which hatched into tadpoles and that the tadpoles when grown lost their tails, grew legs and in turn became frogs. So the belief in spontaneous generation of the larger forms of life disappeared for the most part centuries ago.

In the seventeenth century with the use of the simple microscope and the beginnings of the compound microscope, there came speculation with reference to the origin of living things like molds and other fungi. Hooke in his "*Observation XX. Of blue Mould and of the first Principles of Vegetation arising from Putrefaction*" speculated at length on the origin of this mold and similar forms which he encountered. Among his conclusions were the following (*Micrographia,* p. 127. 1665):

> First, that Mould and Mushrooms require no seminal property, but the former may be produc'd at any time from any kind of *putrifying* Animal, or Vegetable Substance, as Flesh & c. kept moist and warm. . . .
>
> Next, that as Mushrooms may be generated without seed, so does it not appear that they have any such thing as seed in any part of them; for having considered several kinds of them, I could never find any thing in them that I could with any probability guess to be the seed of it, so that it does not as yet appear (that I know of) that Mushrooms may be generated from a seed, but they rather seem to depend merely upon a convenient constitution of the matter out of which they are made, and a concurrence of either natural or artificial heat.

All of which meant, of course, that the author had convinced himself of the occurrence of spontaneous generation under suitable conditions.

As applied to insects and some forms of vermin the idea of spontaneous generation persisted. It was supposed, for example, that when meat was exposed, maggots spontaneously developed from the flesh. In fact, it was not until 1668 that an investigator by the name of Redi hit upon a simple expedient to test the hypothesis. He covered the mouth of a vessel containing meat with a screen and observed that the maggots could develop only from the eggs of flies[3] and never developed when flies were kept away. Soon this type of experimentation and observation was extended so that by the time the microscope had been developed to the point that it was satisfactory for the study of minute forms of life such as the bacteria there was general acceptance of the theory that all living things must come from preexisting parents of the same kind.

But with increased study of bacteria and other microbes the problem of spontaneous generation was again posed. It was readily observed that bacteria swarmed in the presence of decaying organic matter. This observation led students to wonder whether the bacteria did not develop spontaneously from the dead material. To test this theory they boiled meat and various kinds of vegetable matter and found that after such decoctions were kept for a time at room temperatures, the bacteria were present in great numbers. It was assumed that the temperature of the boiling water was sufficient to kill all living things originally present, and hence the bacteria which were found later must have been the result of spontaneous generation. The fact that there are certain types of resistant bacteria (or their reproductive bodies called spores) was not then known, nor that these are able to resist the temperature of boiling water for a considerable period without being killed.

However, Spallanzani (1777) found that bacteria frequently did not develop in food materials which had been boiled for a time and then sealed away from the air. His results were criticized by other investigators, for if correct, they seemed to disprove spontaneous generation. Since air was excluded from his flasks, his opponents contended that the presence of air was essential to spontaneous generation. This argument was met by Schulze (1836), who passed air through sulfuric acid and then into flasks partially filled with boiled decoctions. He noted that the introduction of such air did not induce the development of microorganisms. Schwann in 1837 demonstrated the same fact by passing air through hot tubes. In other words, these

[3] Some flies deposit the living or hatched maggots.

investigators showed that even in the presence of air spontaneous generation did not occur in the boiled food materials. However, even these demonstrations did not entirely disarm those who opposed them. It was contended that heating air before passing it through the cultures "devitalized" it. They believed that air was an essential to spontaneous generation because of its stimulating action on the organic matter with which it came into contact. Those who opposed this theory of spontaneous generation contended that the untreated air when introduced into these flasks caused spoilage because it carried on its dust particles the seed (spores) of microorganisms. In other words, these flasks were seeded with bacteria from the air. These experiments, therefore, did not satisfactorily settle the question as to whether or not spontaneous generation of microscopic organisms did or did not occur.

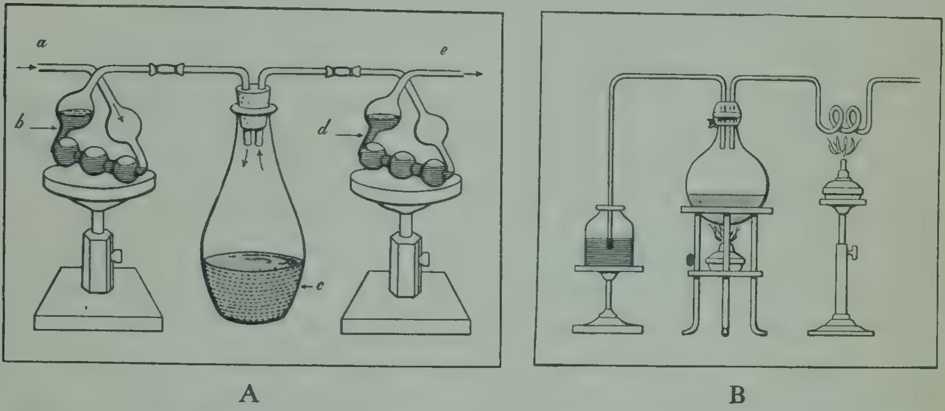

Fig. 1–8. A. Schulze's experiment to determine whether air induced spontaneous generation. The contents of the flask *c* have been sterilized by boiling. Air was passed into the system at *a,* then bubbled through the sulfuric acid in *b* to remove any living matter, then through the flask *c* and thence through the bulbs at *d*. The flask *c* remained without growth. B. Schwann's experiment to detect spontaneous generation. The contents of the balloon flask were heated to destroy microorganisms present, then cooled. The tube to the right was continually heated so that any air entering the flask would be sterilized. No growth occurred.

Three different series of experiments made about the middle of the nineteenth century finally answered all the objections of those who believed in spontaneous generation and proved quite conclusively that life, so far as is known at the present time, must come from preexisting life of the same type, and that there is no such thing as spontaneous generation of microorganisms. Schröder and Dusch in 1854 showed that filtration of air through sterile cotton filters quite completely removed its power of inducing the development of organisms in boiled liquids kept in flasks. It is scarcely probable that the cotton plug to such a flask could act in any other way than as a mechanical filter. It was assumed naturally that it was able to remove all floating particles from the air which gradually passed through it. This demonstration of the efficiency of the cotton filter is the basis for the universal practice in the bacteriological laboratory of protecting sterile materials in test tubes and in flasks by means of cotton plugs.

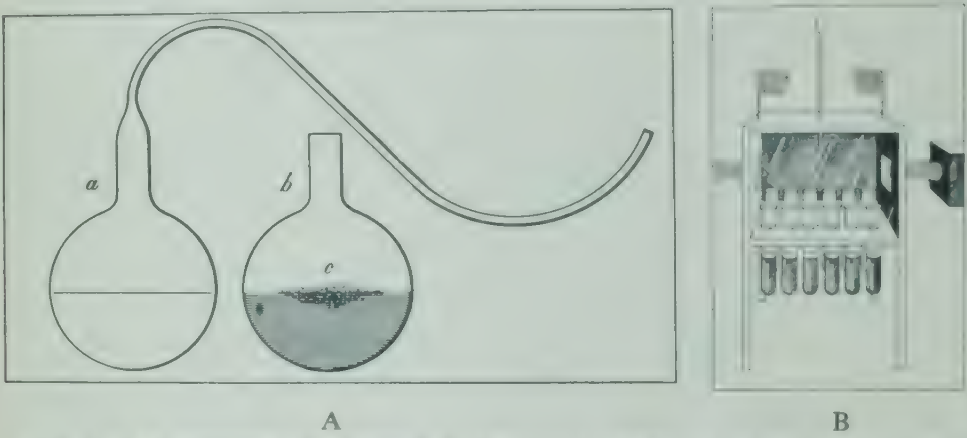

Fig. 1–9. A. Pasteur's experiment to determine whether air induced fermentation. The contents of both flasks were sterilized. The flask *a* remained in contact with the air through the long bent glass tube. Flask *b* was permanently exposed to the air through a large opening at the top. Flask *a* remained sterile, the flask *b* soon showed a luxuriant growth of microorganisms. B. Tyndalls's experiment to determine relationship of dust particles in the air to fermentation of fluids previously sterilized. The box with its glass window was fitted as illustrated with tubes containing a suitable medium for the growth of microorganisms, sterilized by heating. A beam of light passed from right to left through the box showed the presence or absence of dust particles in the air. When dust particles were evident in the air, the contents of the tubes fermented. When sterile media in the tubes were exposed to air in which there were no dust particles no fermentation occurred.

Pasteur in 1860 showed that communication might be maintained between the sterile contents of a flask and the open air by means of a long tube so bent as to minimize the chance of any floating particles of the air gaining entrance and seeding the liquid. Somewhat later Tyndall developed the final proof that the floating matter of the air is responsible for growth in liquids which have been sterilized and then exposed. He showed that sterile liquids exposed to air which had been completely freed of such particles remained without growth.

He first developed a satisfactory test for determining the freedom of the air from dust particles. A beam of light passed through an opening into an otherwise darkened box was visible if there were floating particles; if none were present, the beam was invisible, i.e., he used the principle of observing motes in the sunbeam. The cumulative effect of the studies of these investigators seemed to establish with a high degree of certainty that the doctrine of spontaneous generation of life is false.

A controversy such as that just outlined could not be resolved except by studies in the laboratory. In consequence many new facts were discovered about bacteria and other microorganisms; what they looked like, how they grew and multiplied, and how different kinds could be distinguished. Most important, it was recognized that each kind of organism such as a typhoid germ or a yeast cell must originate from preexisting organisms of the same kind. Such recognition laid a firm foundation for the development of a science of bacteriology.

The Germ Theory of Fermentation and Decay and Its Effect on the Development of Bacteriology. The discovery that many microorganisms are present in fermenting and decaying materials of all kinds led naturally to the question: Are these organisms responsible for the various chemical and other changes going on in fermenting or decaying materials, or are they simply a byproduct, or possibly attracted and nourished by the chemical substances produced quite independently of them? In other words, do the bacterial and yeast cells produce the changes which we call fermentation and decay, or do the chemical changes which take place encourage the growth of these organisms merely by making the conditions favorable for their development?

Two workers, Cagniard-Latour and Schwann, independently in 1837 studied the microscopic granules which developed in all liquids undergoing alcoholic fermentation and concluded that these yeast cells were microscopic plants, and because they were always present in such liquids they concluded that the production of alcohol must be bound up with the growth of the yeasts. However, the idea that microorganisms are incidental and not causal was upheld with great vigor by the celebrated German chemist Liebig. He so dominated the field of chemistry in the period from 1840 to 1860 that relatively few scientists had the temerity to question his conclusions. The French investigator Pasteur, however, from time to time published experimental results from which he concluded that microorganisms must be the primary cause of most types of fermentations. These conclusions were strenuously opposed by Liebig and his school, who ridiculed them as puerile and quite unworthy of serious consideration. Pasteur, however, took the problem into the laboratory and continued to pile up evidence until finally he succeeded in showing quite conclusively that the alcoholic fermentation of liquids containing sugar is commonly due to the presence of yeasts, the souring of milk and the transformation of wine into vinegar are due to the activity of bacteria. Since the time of this controversy, the proofs have accumulated until we now recognize that practically all putrefaction, fermentation, and decay are due to the activity of microorganisms. Furthermore, in most cases it has been possible to isolate and study the particular organisms responsible for each of the changes. These facts aided also in getting a background for the further studies next to be noted on the causal relationships of microorganisms to disease. They also furnished much valuable and important information concerning the transformation of multitudes of organic compounds in nature, concerning the purification of water, the disposal of waste, the enrichment of soils, and the changes both desirable and undesirable brought about in food materials.

By these discoveries, and the laboratory studies which resulted there gradually opened up the whole field of the physiology of the bacteria and other microorganisms. It was found that many physiological processes which could be studied successfully with these minute forms of life were equally significant in higher animals and plants. Many discoveries in the physiology of the bacterial and yeast cell have found application in human physiology. As one noteworthy example, the discovery by Wood and Werkman (1936) that ability to take up and utilize (assimilate) carbon dioxide in the absence of light is a characteristic of many bacteria led promptly to the discovery that this property, so long overlooked, is also possessed by certain cells of the animal body. Furthermore, the recognition that the cells of bacteria, yeasts, and molds are suited to the study of fundamental metabolism, has led to many uses as tools in the laboratories of the biochemist, where he employs them in determination of the constitution, even the presence, of various chemical substances otherwise difficult to detect and to measure quantitatively.

The Germ Theory of Disease—How Its Verification Stimulated Development of Bacteriology. Several times in history suggestions have been made that tiny germs of one kind or another might have some relationship to diseases of man or animals. Certain philosophers of ancient Greece believed that minute worms or germs floating through the air might be responsible for the production of disease. There was, of course, no adequate proof of the validity of these suggestions, and in general they were disregarded until the studies of Leeuwenhoek demonstrated that microorganisms are widespread. Apparently Plenciz (1762) was the first to state clearly in modern times a theory which definitely ascribed disease to the presence of microorganisms. He claimed that each disease was caused by a particular kind of germ and that these germs could be carried from one person to another through the medium of the air. However, the science of bacteriology had not developed to such a point in his day that it was possible to secure any adequate experimental proof for his theories. They were, in other words, wholly speculative.

The first satisfactory demonstration of the probable causal relationship of organisms to disease was made by Davaine in 1863. He found that the blood of animals attacked by the disease anthrax could be shown to contain rod-shaped microorganisms, and that the disease could be transmitted by transfer of such blood to a healthy individual. A similar relationship was found by Pasteur in 1865 when he proved a destructive silkworm disease in southern France to be due to the activities of a protozoan parasite. Somewhat later Robert Koch (1875) was able not only to confirm the earlier results of Davaine with the disease anthrax, but also to grow the causal organism in appropriate nutrients in the laboratory, and to reproduce the characteristic disease in cattle by the injection of pure cultures. In 1882 Koch also introduced liquefiable solid media which greatly simplified the securing of pure cultures of bacteria from the mixtures in which they are generally found under natural conditions.

The use of these methods was soon followed by the isolation and identification of the kinds of microorganisms causing a wide variety of diseases. Techniques useful in staining bacteria to make them more plainly visible under the microscope were also worked out. To these studies we owe the rapid advance which has occurred since about 1890 in our knowledge of the cause of diseases, and the firm establishment of the *germ theory of disease*. This should not be interpreted as stating that all diseases are caused by germs. On the contrary it has made possible the very clear differentiation of diseases into two groups, those caused by microscopic organisms (and called in consequence *infectious*), and those not so caused (or *non-infectious*).

Today we are familiar with the microorganisms which cause a large proportion of the infectious diseases of man and animals. There are, however, some diseases which we believe to be infectious but whose causal microorganisms have not yet been detected. In recent years the greatest increase in our knowledge has come about with development of methods of studying and characterizing the causes of virus diseases. The study of these viruses and their relationships to disease has been greatly complicated by their minuteness. For the most part they are quite too small to be seen by the highest magnifications attainable with the usual laboratory microscope. Some knowledge of their shapes and sizes has been gained by the use of the electron microscope, and by other methods.

The development of the germ theory of disease has quite completely revolutionized much of the practice of modern medicine. It has determined largely the direction and magnitude of growth of modern sanitary science and of preventive medicine. It is evident that when it was found that microorganisms were responsible for many diseases, every effort was made to study these in the laboratory in order to determine methods of destroying these organisms and combatting the diseases which they produced. The modern importance of bacteriology has come about, at least in part, as a result of the development of the germ theory of disease.

CHAPTER 2

Organisms Usually Included in a Discussion of Bacteriology or Microbiology

No English word satisfactorily includes all the forms or groups of microscopic life that are usually studied by the bacteriologist or microbiologist. Five distinct groups are to be considered: the bacteria, the yeasts, the molds, the protozoa, and the viruses. Three different words are in common use as inclusive designations for these forms. The first, *microbe,* comes from the French, and means a small or microscopic living thing. It is less commonly used than the equivalent word *microorganism*. Sometimes the word *germ* is employed, but this has so many other meanings that its use may lead to ambiguity.

Three of the groups listed above, the bacteria, the yeasts, and the molds, belong in the plant kingdom, and the protozoa in the animal kingdom. The position of the viruses is uncertain, some students indeed do not regard them as living organisms. The question arises as to what methods or criteria may be used in the differentiation of plants from animals or, more concretely, the bacteria, yeasts, and molds from the protozoa. The characteristics and relationships of the viruses must also be reviewed critically.

WHY BACTERIA ARE REGARDED AS PLANTS AND NOT AS ANIMALS

The earlier observers of bacteria believed them to be animals rather than plants, largely because many of them were found upon microscopic examination to be able to swim about actively, that is, they were *motile*. Later students concluded on the basis of other characters that they were in fact plants. Why has there been this change in point of view? What are the real differences between plants and animals?

Differentiation between higher plants and higher animals is relatively easy, for their contrasting characteristics are readily observed. But with the microscopic organisms much greater difficulty is often experienced; in fact it is possible to find forms intermediate between plants and animals such as to constitute a nearly complete series of intergradations between the two groups. This difficulty of definite allocation to the plant or the animal kingdom is particularly pronounced with the bacteria, for they are among the simplest known plants and intergrade with some of the simpler single celled animals or protozoa. What are some of the reasons for the inclusion of bacteria among the plants?

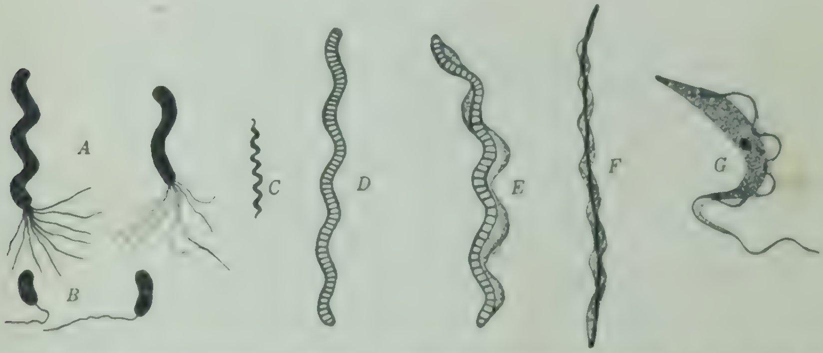

Fig. 2–1. Intergradations between bacteria and protozoa. A–B, bacteria of genera *Spirillum* and *Vibrio;* C–F, intermediate forms; C, *Treponema;* D, *Saprospira;* E, *Cristispira;* F, *Spirochaeta;* G, protozoan, *Trypanosoma.*

There is no single or simple test or criterion for distinguishing bacteria from protozoa. Usually a plant cell is surrounded by a relatively firm and well differentiated cell wall, quite distinct from the living cell contents or protoplasm. Such plantlike cell walls may usually be demonstrated in bacteria. Definite cell walls are not commonly produced by animal cells, the bounding surface is less definitely differentiable from the cell contents. However, protozoa sometimes go into a resting stage in which they produce a definite cell or cyst wall. Furthermore, the bacterial cell walls resemble in composition those of certain other organisms classed as plants (as the fungi and algae), though the walls of all these usually differ in chemical composition from those of higher plants. The character of the bacterial cell wall on the whole is definitely plantlike.

The cells of most bacteria (not all) are relatively stiff, they do not flex when the organism moves, the cell does not change shape. On the other hand many protozoa readily change their shapes. Certain bacteria, however, do bend the cell, in fact some are able to swim by means of rapid cell flexion. In such cases the distinction between motility of bacteria and protozoa tends to break down, and such flexuous bacteria are by some authors included with the protozoa, or are regarded as intermediate forms. One bit of evidence tending to group even these intermediates as bacteria is that the cells when they increase in numbers divide transversely, while the protozoa which most closely resemble them divide lengthwise. Figure 2–1 illustrates certain bacteria which are typical, some which may be regarded as intermediate, and a true protozoan.

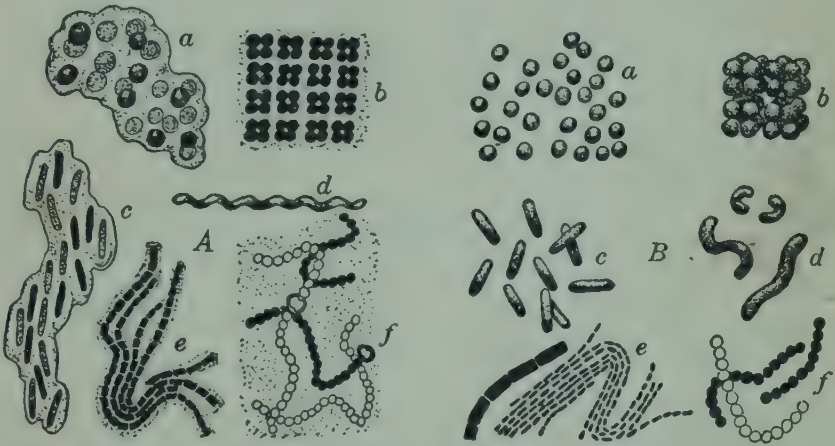

Fig. 2–2. To illustrate the apparent relationship of the bacteria to the blue-green algae. Those forms most closely resembling each other are lettered alike. A. blue-green algae: *a, Aphanocapsa; b, Merismopedia; c, Gleotheca; d, Spirulina; e, Phormidium; f, Nostoc.* (All adapted from West.) B. bacteria: *a, Micrococcus; b, Sarcina; c, Bacillus; d, Spirillum; e, Bacillus* in chains; *f, Streptococcus.*

A still more cogent reason for regarding bacteria as plants is that there are very many evidences of kinship to other unicellular forms quite generally regarded as plants. The relationship of certain groups of bacteria to the blue-green algae is particularly close. Figure 2–2 illustrates the resemblance of these groups morphologically. In many cases it would be quite legitimate to regard the bacteria as algae which had lost their green color (chlorophyll). Some bacteria possess pigments which function somewhat as does chlorophyll in the green plants in enabling the cells to utilize the energy of light in building foods from carbon dioxide. Such forms show very close affinities to the algae. Other groups of the bacteria show almost every intergradation with certain of the fungi (a group classified with plants), both in shape and branching of cells and in reproduction. This confirms our allocation of the bacteria to the plant kingdom.

The fact that many (not all) bacteria are motile, swimming about actively in water by means of organs of locomotion (called flagella), should not be regarded in any sense as characterizing them as animals. We are so accustomed to seeing the higher plants anchored in place and the higher animals moving about that we are likely to associate motility only with animals. This is quite unwarranted, for many typical plant cells are known to be able to swim about, among them many of the algae, some of the fungi, and certain of the reproductive (sperm) cells of other plants, such as mosses, ferns, and even some seed plants (as the gingko or maiden-hair tree). It is well to note also that some animals are non-motile, at least at certain stages in their life cycles. Figure 2–3 illustrates the fact that organs of motion and motility are not exclusive characteristics of either animals or plants.

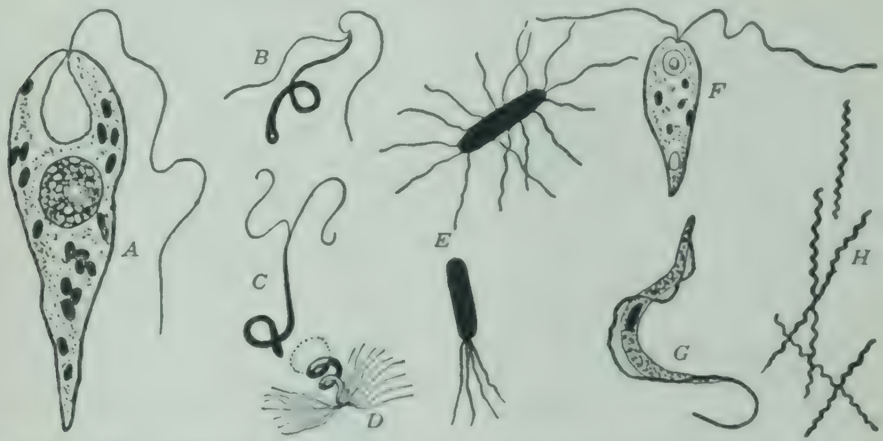

Fig. 2–3. To illustrate that cell motility may be characteristic of both plant and animal cells. A, *Euglena*, a motile unicellular green alga; B, *Chara* sperm cell, a motile algal cell; C, motile sperm cell of a moss; D, motile sperm cell of a fern; E, motile bacterial cells; F, a motile protozoan from stagnant water; G, a motile parasitic protozoan, *Trypanosoma;* H, a motile flexuous bacterial cell, *Treponema*.

PLACE OF BACTERIA, YEASTS, AND MOLDS IN THE PLANT KINGDOM

It was previously noted that the plantlike organisms to be considered under the heading of microbiology (or of bacteriology in a broad sense) are the bacteria, the yeasts, and the molds. Just where do these forms belong in the plant kingdom?

Plants may be classified into four great groups: the *Spermatophyta* or seed plants, the *Pteridophyta* or fern plants, the *Bryophyta* or moss plants, and the *Thallophyta* or thallus plants. The last group is the one that includes the bacteria, yeasts, and molds as well as many other subdivisions. The *Thallophyta* are differentiated from all other groups of plants by the lack of a complex plant body. Sometimes the plant consists of but a single cell, frequently of a thread made up of cells united end to end, but there are never formed complex plant tissues with differentiation into roots, stems, and leaves.

The group *Thallophyta* is a very large one, containing tens of thousands of species. It may be divided into three subgroups: the *Schizophyta* or fission plants, the *Algae*, and the *Fungi*. The members of the group *Schizophyta* are distinguished from all other plants by being unicellular, and by having fission (splitting or dividing at right angles to the long axis of the cell) as a common method of multiplication. This group is divided into two classes, the *Schizophyceae* or blue-green algae, and the *Schizomycetes* or bacteria. The cells of the former contain chlorophyll or "leaf green," while the cells of the bacteria are devoid of green pigment (except for a few intermediate forms). The bacteria are included among the forms to be considered in this text.

The group *Algae* includes the seaweeds, pond scums, water-silks, and similar forms. None of them are considered further here. The group *Fungi* differs from the *Algae* in the absence of chlorophyll and includes the toadstools, puffballs, mushrooms, smuts, rusts, mildews, yeasts, and molds. Only the two groups last mentioned are included in the scope of microbiology or bacteriology as considered here.

The following classification will assist in getting a clear idea of the position of the plant groups to be considered. Those to be discussed are listed in boldface type, the others in italics.

Thallophyta (thallus plants). Simple, relatively undifferentiated plants. Never producing roots, stems, leaves, flowers or seeds.

- **Schizophyta** (fission plants). Simple, unicellular plants usually multiplying by dividing of cells.
    - *Schizophyceae.* The blue-green algae, containing chlorophyll.
    - **Schizomycetes.** The bacteria, not containing chlorophyll.

Algae, more complex forms containing chlorophyll, including seaweeds, pond scums, water-silks, etc.

Fungi, more complex forms, without chlorophyll.

Yeasts. Unicellular, usually multiplying by budding.

Molds. Multicellular, also the *Mildews, Smuts, Rusts, Mushrooms, Puffballs*, etc.

DIFFERENTIATION OF BACTERIA, YEASTS, AND MOLDS

Criteria for the differentiation of the bacteria, yeasts, and molds should be understood. In general there is little difficulty in telling the three groups apart, though occasionally intergrading or intermediate forms are encountered which prove somewhat troublesome.

Molds are multicellular, bacteria and yeasts are unicellular. While bacterial or yeast cells may adhere in masses, groups, or chains, the individual cells are independent plants; in the mold many cells are united to form a filamentous plant body.

The differentiation of yeasts and bacteria is somewhat more difficult. The following are the more easily recognized differences:

1. In general, yeast cells are somewhat larger than bacterial cells. This is not always true, but the statement holds for most of the bacteria and yeasts commonly studied in the laboratory. Facility in differentiation on the basis of size can result only from experience in observation.

2. Most bacteria multiply by transverse fission (splitting) of the cell, most yeasts multiply by a process of budding. This is usually the easiest method of differentiation, it holds good generally for the bacteria and yeasts commonly cultivated in the laboratory. A few kinds of yeasts, however, are known which multiply like bacteria, by fission. Furthermore, some types of bacteria, particularly in old cultures, may produce buds, or multiply by still other methods. The method of differentiation is, however, sufficiently accurate so that the names *Schizomycetes* (fission fungi) and *Blastomycetes* (budding fungi) have been applied to the bacteria and yeasts respectively.

3. A criterion which frequently proves helpful is differentiation on the basis of the changes produced by the organisms when grown under suitable conditions. Most (not all) of the common yeasts are active in the fermentation of sugar to alcohol and carbon dioxide. While some bacteria may produce small amounts of alcohol in their growth, in general alcoholic fermentation is due to yeasts.

Other differences between yeasts and bacteria and between these and molds will become evident as these groups are discussed, and their morphology and physiology are studied.

THE PROTOZOA

The protozoa constitute the fourth group (in addition to the bacteria, yeasts, and molds) frequently considered in microbiology. The study of the protozoa in reality constitutes a distinct subdivision (*protozoology*) of the science of zoology. The term *protozoa* includes all unicellular animals. Most protozoa are not causally related to chemical and fermentative changes of economic significance. Some are pathogenic (disease producing) and are studied most effectively by the same techniques as have been developed for a study of the bacteria. A few forms are important in soils, in water, and in sewage, others are significant in the digestion of food by certain animals, such as the ruminants. Still others have found significant use in the laboratory in studies of physiology and biochemistry. Several of the pathogenic forms will be discussed in a consideration of the role of microorganisms in production of specific diseases.

The criteria which may be most useful in differentiating protozoa from bacteria are the following:

1. Protozoa belonging to one of the great groups show a peculiar type of motility not found among the bacteria. The cells are able to change shape rapidly, they flow about. This type of motion is termed *ameboid* (from *ameba,* an organism which shows this type of motion particularly well).
2. In several of the great groups of protozoa solid food particles are taken into the interior of the cell (ingested) and there digested. Bacteria and other protozoa absorb only food material in solution.
3. Very few of the protozoa grow in chains or filaments as do the molds and certain of the bacteria.
4. Many of the pathogenic protozoa have relatively much more complicated life cycles than do the bacteria. This will become evident from a study of the parasitic forms.
5. In general protozoa do not show a well-defined wall while active, though when they go into a resting stage (encyst) a definite wall is evident.

By use of appropriate staining methods it is usually possible to show that the protozoan cell contains a definite nucleus.

THE VIRUSES

The viruses differ most strikingly from the bacteria, yeasts, molds, and protozoa in their extreme minuteness. In general they are so small as to be invisible even under a powerful microscope, in a few cases they are barely visible. They are therefore usually said to be ultramicroscopic and filterable. The latter term indicates that the particles of virus are small enough to pass through a filter in which the pores are sufficiently small to hold back ordinary bacteria. It will be noted that the term *particle* has been used instead of microorganism or bacterium in characterizing the viruses. The reason is that there is not complete agreement as to whether the particles are living, or whether they are closely related to or possess some of the characteristics of an enzyme, or are huge protein molecules. Certain viruses such as that of the plant disease tobacco mosaic may be purified and even crystallized. In a few cases virus particles are just at the lower limits of microscopic visibility, and do not differ markedly from other microorganisms except in size. A more complete account of the characteristics of the viruses will be given later (Chapter 6).

The viruses[1] are of considerable significance, as they are the cause of many diseases of plants and animals and bacteria. So far as is known, all are parasitic within the tissues of plants or animals, usually within the cells. A virus which attacks bacteria is termed a *bacteriophage,* or more briefly, a *phage*.

[1] The word *virus* comes directly from the Latin, in which one of its meanings was a "poison," or "venom." It is one of the half dozen Latin words which end in *-us* and are neuter. There seems to be no example in Latin literature in which the word was used in the plural, nor is there any certain rule to tell what the plural should be. Holmes on the basis of analogy and after consultation with philologists concluded that the plural should be *vira*, and recognized *Vira* as a class of microorganisms. The usual English plural is *viruses* though *vira* may be used.

CHAPTER 3

How Microorganisms Are Named and Classified

Plants and animals generally, including those studied in microbiology, have two kinds of names applied to them. The first kind is the common name used in the language of each country. In English we use the name oak for a certain group of trees; the German equivalent is *Eiche,* the French is *chêne,* the Swedish *ek,* the Greek *drys,* the Latin *quercus.* Sometimes a plant or animal may have several common names in the same language, the plant known as alfalfa in the United States is termed lucerne in England. Names of this type peculiar to a language or dialect are sometimes termed trivial, casual, or vernacular names. Sometimes such names are used conveniently and appropriately in bacteriology. For example, a microorganism frequently causing pneumonia is often called the *pneumococcus,* that which causes a type of meningitis the *meningococcus,* that which causes tuberculosis the *tubercle bacillus.* The use of such names is often in the interest of convenience and brevity.

To each kind of living thing it is desirable that there be given a technical or scientific name which will be recognized by all scientists as an international or universal designation. Rules for the making and use of such scientific names have been developed by those working in the biological sciences, including botany, zoology, and bacteriology. It was early agreed that these scientific names should be written in Latin and treated as Latin words. The Latin language at the height of the Roman empire was known from Britain to India. It became the official language of the western church, it was widely used in diplomatic and scholarly circles. It became the recognized international language of science. Only within the last two centuries have scientists used languages other than Latin in describing their discoveries. Those working in the fields of botany and zoology gave Latin names to the plants and animals they described. It was not, however, until the time of the Swedish naturalist Carl von Linné (usually written in latinized form as Linnaeus) about the middle of the eighteenth century that a reasonably stabilized method of constructing names for all living things was developed. At the University at Uppsala in Sweden, he with his students took upon himself the task of giving properly constructed Latin names to all the known and the thousands of newly discovered plants and animals. The rules for naming plants and animals have since the time of Linnaeus been in general agreed upon and codified at international congresses of botanists and of zoologists called in part for this purpose.

At an International Congress for Microbiology held in Paris in 1930 a Committee on Bacteriological Nomenclature was set up. At this and subsequent meetings it was directed to use the botanical and zoological rules of nomenclature as models and to develop from them a set of rules (Nomenclatural Code) which would have such modifications as would particularly fit bacteriology. The code for bacteriology was finally completed by the committee and approved by the International Congress of the Association of Microbiologists at Copenhagen in 1947. This then becomes the authoritative set of rules which will be followed by bacteriologists generally.[1]

Unfortunately some researchers in the field of microbiology who have made substantial contributions to the science have not understood or at least have not used the older botanical and zoological codes; there exists therefore in the literature of bacteriology considerable lack of uniformity in the names applied to the bacteria. The student is not to be surprised therefore at the differences to be found in articles written by different authorities and in the usages in different texts on bacteriology. An "official" list of names has never been compiled. This is a task that has been delegated to a Judicial Commission and is under way. For the most part the names and classifications given in the last edition (sixth) of Bergey's *Manual of Determinative Bacteriology* are used in this text, but some exceptions are made. Where in the opinion of the author another name is more nearly correct, it will be employed with a brief explanation[2] of the reason

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[1] Those who are to be professionally engaged in bacteriology should be familiar with the provisions of the code. It constitutes a guide to one who finds it necessary to name new species or to determine the correctness and validity of the names given by others. The Code is printed in *Journal of Bacteriology*, **55**, 287-306 (1948). It should be consulted for details.

[2] The guide most frequently followed in the United States in bacteriological nomenclature is Bergey's *Manual of Determinative Bacteriology*. Its position should be clearly understood. Its Board of Editors is self constituted and self perpetuating

Here are some of the general rules and recommendations common to all fields of biology, together with a few minor differences between the rules for zoology on the one hand and of botany and bacteriology on the other which may be noted.

1. *Words used in scientific names.* The scientific names of all plants and animals, including all bacteria and other microorganisms are in Latin. However, many of them cannot be found in a standard Latin dictionary, for the Latins did not know or recognize many of the plants and animals and certainly not the microorganisms with which we are familiar. Most of the names are newly made, constructed in general according to the rules laid down by the Latins for coining new words. Tens of thousands of modern Latin words have been thus developed. Many of them have been made by changing the spelling of Greek words to conform to Latin rules. For example the Greek *bakterion* has become the modern Latin *Bacterium, kokkos* has become *coccus.* In some cases modern names have been made into modern Latin names: *Pasteurella, Salmonella,* and *Listeria* are modern Latin words formed in honor of the scientists Pasteur, Salmon, and Lister. Two kinds of words are used, nouns and adjectives (or words such as participles used as adjectives).

2. *The scientific name.* Every kind of living thing which has been studied and adequately described should be given a Latin scientific name made up of two words, the first a noun and the second a modifier. For example the name of the microorganism which causes the disease tuberculosis is *Mycobacterium tuberculosis,* that which produces nodules on the roots of certain leguminous plants is *Rhizobium leguminosarum,* one which causes a dysentery is *Shigella dysenteriae,* one present in purulent wounds is *Micrococcus aureus,* the cause of the disease anthrax is *Bacillus anthracis,* of bubonic plague *Pasteurella pestis,* of one type of enteritis *Salmonella enteritidis.*

The first of the two words which together constitute the name of an organism is the name of the group or *genus* (plural *genera*) to which it belongs. This generic name (always Latin or modern Latin) is re-

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Because, however, of the excellent work manifested in the successive editions, it is rapidly assuming the position of a standard reference for determining the names to be used for the various kinds of bacteria. It should be familiar to every student of bacteriology.

Some confusion has developed due to the fact that the editorial board of the first edition of Bergey was recognized as a committee of the Society of American Bacteriologists. There is no such present relationship, and neither the names nor the classifications are to be regarded as approved or official.

garded as a proper noun and is *always* to be spelled with a capital letter. In the examples given in the preceding paragraph the generic names have been made in different ways. *Bacillus* is a classic Latin word, meaning little rod or staff. The ending *-illus* indicates that it is a diminutive. *Listeria* is a Latinized generic name derived from that of Lister, the eminent bacteriologist and surgeon. A generic name may be made of the name of a person by adding *-ia*. Sometimes it is made by adding the diminutive ending *-ella*, as in *Pasteurella* from Pasteur and *Salmonella* from Salmon. The generic name *Mycobacterium* is a modern Latin word made by combining the stem, *myc-* of the Greek word which means fungus (*mycēs*) and that of the Greek word which means little rod (*bacterium*) with the connecting letter *o* used by the Greeks when they made compound words. The word *Mycobacterium* means then a little fungus rod. The ancient Latins and Greeks didn't know anything about microscopes or microorganisms. It is not surprising therefore that it has been necessary to make new words to name most of the many genera of bacteria and other minute forms of life.

Each generic name is usually the name of a group of related kinds. Each kind of plant or animal or microorganism is termed a *species* (plural, *species*). The name of the kind or species is formed by the addition of a second word to the genus name, this second word is called the *specific epithet*. The name of the *Mycobacterium* which causes the disease tuberculosis is *Mycobacterium tuberculosis*. Note that the two words together make the species name. The specific epithet always follows the generic name and modifies it. All words used as specific epithets in scientific names are made into modern Latin words if not found in classic Latin. Just how is this done?

Take first the specific epithet *tuberculosis* as used above. The Latins used the word *tuber* for a swelling, lump, or tumor. They added a diminutive ending *-culum* forming the word *tuberculum* meaning a small swelling or nodule. Then the modern Latin word was formed from the stem *tubercul-* by adding *-osis* which generally conveys the meaning of "disease characterized by the presence of." So *tuberculosis* (adopted also in English) is a modern Latin word meaning disease characterized by nodules or tubercles. In the species name *Mycobacterium tuberculosis* the specific epithet is a noun in the genitive (possessive) case modifying *Mycobacterium* and meaning the *Mycobacterium* of tuberculosis.

Many examples of nouns used in the genitive as specific epithets may be found among the bacteria. For example, *Escherichia coli* means *Escherichia* of the colon or large intestine, *Clostridium welchii* the Welch's *Clostridium,* and *Rhizobium leguminosarum* the *Rhizobium* of leguminous plants.

Occasionally the specific epithet is a noun used in explanation of (in apposition with) the generic name; it is not then put in the genitive case. In English one may use such an expression as George the king (*Georgius rex*). So such names as *Bacillus radicicola,* meaning *Bacillus* the root dweller have been constructed.

Most commonly the specific epithet is an adjective or a participle used as an adjective. For example *Bacillus subtilis* means the slender *Bacillus, Sarcina lutea* the yellow *Sarcina,* and *Pseudomonas fluorescens,* the fluorescing *Pseudomonas.* One must use care to see that an adjective employed as a specific epithet agrees in gender with the generic name which it modifies.

In summary, the specific epithet of a species name may be a noun in the genitive, a noun in apposition or an adjective which modifies the generic name and agrees with it in gender.

This system of giving names made up of two Latin (or modern Latin) words was first definitely proposed by Linnaeus and used by him in the decade 1750–1760. It is termed the binomial system of nomenclature. It should be emphasized that a species name may properly consist of two words only, that is, trinomials and polynomials are invalid. Some bacteriologists have ignored the binomial rule and have endeavored to use an entire Latin descriptive phrase, such as *Bacillus membranaceus amethystinus mobilis,* meaning the *Bacillus* which produces a membrane when growing, develops an amethyst color and is able to swim. Such a name is completely invalid. The coining or use of such names is clear evidence of lack of adherence to the well-recognized rules of nomenclature.

However, there are two types of cases in which three Latin words may be used legitimately in a scientific name. Sometimes more than a single word is required to express a single idea. For example the English epithet lactic acid consists of two words, yet it is the name of a single substance. In Latin the epithet is *acidum lacticum.* Suppose that one wishes to name an organism, the *Bacillus* of lactic acid, it would be *Bacillus acidi lactici.* In such cases, where the specific epithet is made up of two words expressing a single idea, it is customary to join them with a hyphen, so that properly in bacteriological literature the name would be written *Bacillus acidi-lactici,* or perhaps (and properly) the two words are written solidly, and the name becomes *Bacillus acidilactici*. Such a case therefore does not constitute an exception to the rule. One other apparent exception to the binomial rule develops out of the fact that species of organisms sometimes have subdivisions called subspecies or varieties. Suppose, for example, in studying the organism which causes the disease bubonic plague, *Pasteurella pestis*, one finds a variety which is typical in all its characteristics except that it produces a golden yellow color when grown in the laboratory and decides that the variety should have a name. Such a name might well be *Pasteurella pestis* variety *aureus*, or abbreviated, *Pasteurella pestis* var. *aureus* or still more abbreviated *Pasteurella pestis aureus*. As last written, the name is a trinomial, but it is the name of a *variety* and not of a species. It is permissible, therefore, to use trinomials in naming a variety, but not in naming a species.

Higher Groups.[3] Not only are species grouped into genera, but genera are in turn combined into related groups termed families. The name of the family is usually derived from that genus which is most characteristic, called the type genus. Family names of plants and bacteria are formed by adding the ending *-aceae* to the name of the selected genus. For example, the rose family in botany is named from the genus *Rosa*, the *Rosaceae*. The ending *-aceae* is plural and means *resembling or having the characteristics of*. The family *Rosaceae* then includes the genera of plants that sufficiently resemble *Rosa* (or rose). This does not mean that the other genera in the family are like *Rosa* in all or even in many characteristics, but all of them do sufficiently resemble it to be grouped together with it. For example plants as unlike as roses, apples, hawthorns, and strawberries represent different genera belonging to the *Rosaceae*, but they are not all roses. So in bacteriology the genus *Bacillus* is the type genus of the family *Bacillaceae*, which includes also the genus *Clostridium*.

The family names of animal forms (including, of course the protozoa) end in *-idae*. For example, several genera of protozoa which have certain characters in common with the genus *Amoeba* are placed together in the family *Amoebidae*.

Related families are grouped together into an *order*, and related orders into a *class*. The name of an order in botany or bacteriology may be recognized by the ending *-ales* as in the order *Actinomycetales* which includes the family *Actinomycetaceae*.

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[3] Any group of organisms, a species, a genus, a family may be designated as a *taxon* (plural *taxa*).

Sometimes intermediate groups are recognized between genera and families and are termed tribes and subtribes. Similarly a subfamily may be placed between family and tribe, a suborder between order and family, and subclass between class and order.[4]

How May One Determine the Correct Name? In many cases several or even many different names have been applied by different workers or authors to the same kind of organism. This is, of course, quite confusing. How may one determine which is right or best?

It should be recognized that it is sometimes necessary to change names of organisms when new facts are discovered. A completed and wholly satisfactory set of names and an adequate classification can be attained only when we can know all the characteristics of all the kinds of living things. In general, however, the rule for determining the correct name to use for an organism is to use the *oldest* name that is in conformity with the international rules and at the same time represents modern knowledge of the group. But in the last analysis the judgment of the individual who is a specialist in the group and who endeavors to follow the rules is in general a good guide. But the student need not expect uniformity on the part of all workers. For example, there are competent bacteriologists who define the genus *Bacillus* as containing all the species of rod-shaped bacteria which produce spores, many others (probably a large majority) prefer to include in the genus *Bacillus* only those rod-shaped spore-bearing rods which will grow in the presence of free oxygen, and place in another genus *Clostridium* those species which will not grow in the presence of air. Which position is to be assumed is a matter of personal judgment. There are no officially or internationally approved names as yet. For the student who is not completely conversant with all the rules it is best to use the names as they are given by some standard manual, such as that of Bergey for the bacteria. This will eliminate much confusion.

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[4] In bacteriology the following endings are used for the various groups, from species to class.

Group	*Ending*	*Example*
Class	No uniformity (plural)	*Schizomycetes*
Subclass	No uniformity (plural)	———
Order	*-ales* (plural)	*Eubacteriales*
Suborder	*-ineae* (plural)	*Eubacteriineae*
Family	*-aceae* (plural)	*Bacillaceae*
Subfamily	*-oïdeae* (plural)	*Bacilloïdeae*
Tribe	*-eae* (plural)	*Bacilleae*
Subtribe	*-inae* (plural)	*Bacillineae*
Genus	A proper noun (singular)	*Bacillus*
Species	A binomial	*Bacillus subtilis*

CHAPTER 4

Bacteria, Their Morphology and Classification

MORPHOLOGY OF BACTERIA

To know the bacteria it is necessary to study their size, shape, arrangement and grouping, cell structure, cell contents, capsules and sheaths, their various organs (organella) including organs of locomotion, spore formation and life cycles, all included under the heading of morphology.

Measurement and Size of Bacteria. The unit commonly used in measurement of objects in the size range of the bacteria is the *micron*, usually abbreviated as the Greek letter μ (mu). A micron is the one thousandth part of a millimeter (the millionth part of a meter). For measurements of objects still smaller than bacteria, the unit employed is the *millimicron* (abbrevated as mμ), the thousandth part of a micron. This latter unit is frequently used to measure ultramicroscopic particles such as the viruses. A still smaller unit, the Ångstrom (the tenth part of a mμ) is also employed for very minute objects, and for certain physical measurements as in recording the wavelengths of light.

Bacteria show wide variations in size. Some are so minute as to be visible only under the highest magnifications of our microscopes, which means that they may be as small as 0.1 μ in diameter. A few species are large enough so that they may be seen with a simple hand lens, that is, there are some which are as much as 10–12 μ in diameter and 40–50 μ in length. However, most of the bacteria which are commonly observed and studied in the laboratory are between 0.5 μ and 2 μ in diameter and from 1 μ to 5 μ in length.

The minuteness of these bacterial cells deserves some special emphasis. An "average" bacterial cell may have a volume of 1 cubic micron (1.0 μ^3). One trillion (one million million) such cells would fill a volume of 1 milliliter (1 cubic centimeter). Bacterial cells may therefore exist in great numbers in materials such as souring milk and yet occupy only a small fraction of the total volume. If, for example, there were present in such milk a million "average-sized" bacteria per milliliter, only one millionth of the volume would be occupied by bacterial cells. Bacteria frequently increase in numbers under favorable conditions until hundreds of millions or even several billions may be found in each cubic milliliter of a fermenting liquid.

Bacteria may show considerable apparent variation in size when examined under different conditions. They are commonly dried and stained before being studied under the microscope. During the process of preparation the cells may show much shrinkage. It is therefore quite necessary that the conditions under which measurements are made should be carefully specified.

Shape of Bacterial Cells. Bacterial cells are usually one of three shapes: *spheres, straight rods,* or *bent rods.* A rod, if sufficiently elongate, is said to be *filamentous.*

A spherical or ellipsoidal bacterial cell is usually termed a coccus.[1] Considerable variation in shape is found among the cocci, although the cells are typically globular. When two cocci lie side by side, the approximated sides may be flattened, sometimes even concave. Such a coffee-bean shape is characteristic of the gonococcus. Another type of coccus with cells usually in pairs has one side of the cell flattened and the other somewhat pointed. Isolated cells in most cases, however, assume a spherical shape.

The cells of many bacteria are rod-shaped and relatively straight. Such a rod-shaped organism may be called a bacillus.[2] The rod may be long or short; it may have rounded ends, or they may be truncate, or even concave.

Some bacteria have the shape of a bent rod. The amount of bending may be slight, the cell having the appearance of a short arc of a circle. Or the cell may be in the form of a spiral, or helix. Such an organism is called a spirillum.[3] It may be slender or relatively thick, short, or long. The cell may show several or numerous complete turns of the spiral, or it may appear merely as a slightly bent rod.

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[1] *Coccus* (plural *cocci*) comes from the Greek *kókkos* which had two principal meanings, a grain or seed, and the globular body of an insect which grows on certain oaks in southern Europe and from which a scarlet dye was produced. So sometimes the scarlet color itself was called *kókkos.* Because of the spherical body of the insect and the shape of many seeds, the word coccus has generally the meaning of spherical. This is the connotation in compound words such as the generic names *Micrococcus* and *Streptococcus.*

[2] The Latin *bacillus* (plural *bacilli*) means a small staff. Mueller named an organism, which was probably a bacterium, *Vibrio bacillus.* Later Cohn (1875) used *Bacillus* as the generic name for certain rod-shaped bacteria. Still later the name was used to apply to any rod-shaped organism. It was also adopted as an English word. It is now used (*bacillus,* not capitalized) as a common name for any rod, and *Bacillus* as the technical generic name for a limited group of rod-shaped bacteria which produce spores. The uses of the two words should not be confused.

Some kinds of bacteria show differences in cell shape, depending upon the stage of the growth cycle in which they are examined. Some are rod-shaped at one stage and spherical at another. Some also change their shape when placed under new and different environmental conditions.

Arrangement and Grouping of Bacterial Cells. Bacteria usually multiply by splitting crosswise, i.e., by transverse fission. In some cases the daughter cells separate promptly, in other cases they remain united for various periods of time. When they remain attached, cell groups are formed which may be characteristic and useful in identifying the organism. The shape of the group of cells thus formed and the cell arrangement within the group are determined largely by the manner of multiplication and the direction of the planes of division.

The cocci show great diversity in cell arrangement. It is a general rule among bacteria that the wall that divides a cell into daughter cells is formed at right angles to the longest axis of the cell. Since a sphere has no longest axis, cell division in cocci may occur in any direction. The daughter cells may immediately separate after the completion of the division. This results in the development of isolated cells only. With most forms, however, the cells remain together, for a time at least. A coccus in which the successive planes of division are constantly parallel forms, as a result, rows of cells resembling a chain of beads. This type is known as a streptococcus.[4] In some forms the cells remain typically united in pairs; one of this type is termed a diplococcus.[5] When the cells remain attached in irregular masses, resembling clusters of grapes, the name used is staphylococcus.[6] This last grouping usually results from irregular

[3] The common name (not capitalized) *spirillum* (plural *spirilla*) may be used for any spiral (helicoidal) rod. However, the generic name (capitalized) *Spirillum* is used for a specially defined, limited group of such spiral organisms (*a genus*).

[4] The name *streptococcus* comes from a Greek word *streptus* (*streptos*) meaning *easily twisted* or *pliant*. The reference is to the fact that like a necklace the chain of cells is rarely straight. Later it will be noted that most cocci (not all) which grow in chains are placed in the genus *Streptococcus*.

[5] The Greek *diplus* (*diplous*) means *double*. There is also a genus *Diplococcus* which includes some, but not all, of the diplococci.

[6] From the Greek *staphyle*, a bunch of grapes. A genus *Staphylococcus* is sometimes recognized.

division of the cells, the planes of division being at all angles to each other. A sarcina[7] is a coccus which divides successively in three planes, each of the planes being perpendicular to the other two. The cells cling together in cubes or packets, made up of eight cells or of multiples of eight. The groups are sometimes likened in appearance to miniature cotton bales. These groupings and coccus shapes are illustrated in Fig. 4–1.

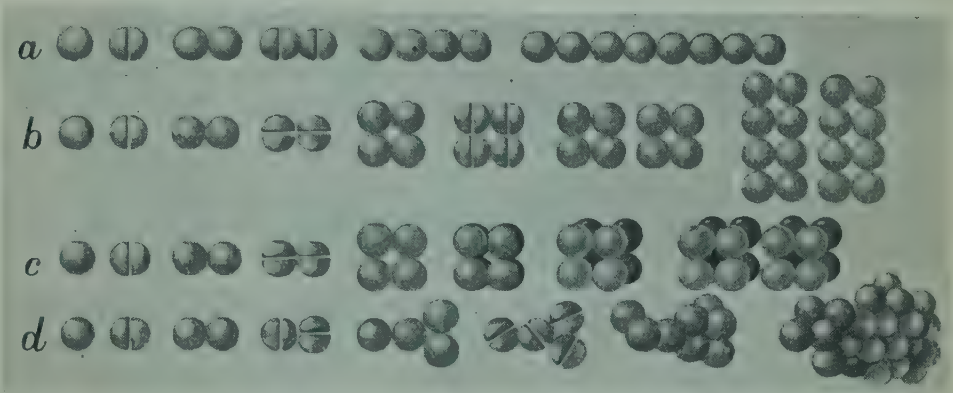

Fig. 4–1. Diagrammatic illustration of the development of the characteristic groupings of the cocci. *a*. Division of the cells in parallel planes, resulting in chains of cells. Organisms thus grouped are termed streptococci. *b*. The cells divide in two planes alternately, at right angles, forming plates of cells, squares or tetrads. They may be designated as tetracocci. *c*. The cells divide successively in three planes, each perpendicular to the other two. The cells are grouped in cubes or regular packets. They may be termed sarcinae. *d*. The planes of division of the cells are not at right angles, the cells are arranged in irregular masses. They may be termed staphylococci.

Bacilli may separate at once after cell division, or they may remain united in chains. Inasmuch as bacilli never divide longitudinally, other groupings are not common.

Spiral cells or spirilla are generally separate, although occasionally they may occur in chains.

Rod-shaped bacteria may exhibit special groupings, or arrangements, of cells in young colonies due to the manner in which they multiply. Graham-Smith divides the rods into four groups on the basis of their movements following cell fission. The first group (*loop-forming group*) includes bacilli (like the anthrax bacillus) which grow in chains, and in which the cell membranes do not break apart upon formation of new cells. As each new cell continues to grow in length it presses against the ends of the adjoining cells, with a consequent tendency for the chains of cells to bend and produce loops. A second group (*folding group*) includes forms which grow in chains with the cells less firmly attached, the cells tend to form zigzag patterns. In the third (*snapping group*) the membrane connecting two cells suddenly ruptures at the side under tension, and the daughter cells remain attached at a corner giving rise to an angle. Those belonging to the fourth (*slipping group*) break apart readily; the cells as they grow tend to slip past each other and form parallel lines. These different ways in which the rod-shaped bacteria may behave during multiplication cause differences in the appearance of masses of these bacteria when growing together (in colonies) and are in part the basis upon which differentiation into rough (R) and smooth (S) colonies (to be described later) may be made. (See Fig. 4–2.)

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[7] The word *sarcina* (plural *sarcinae*) is from the Latin, meaning a package or bundle. There is also a generic name *Sarcina*.

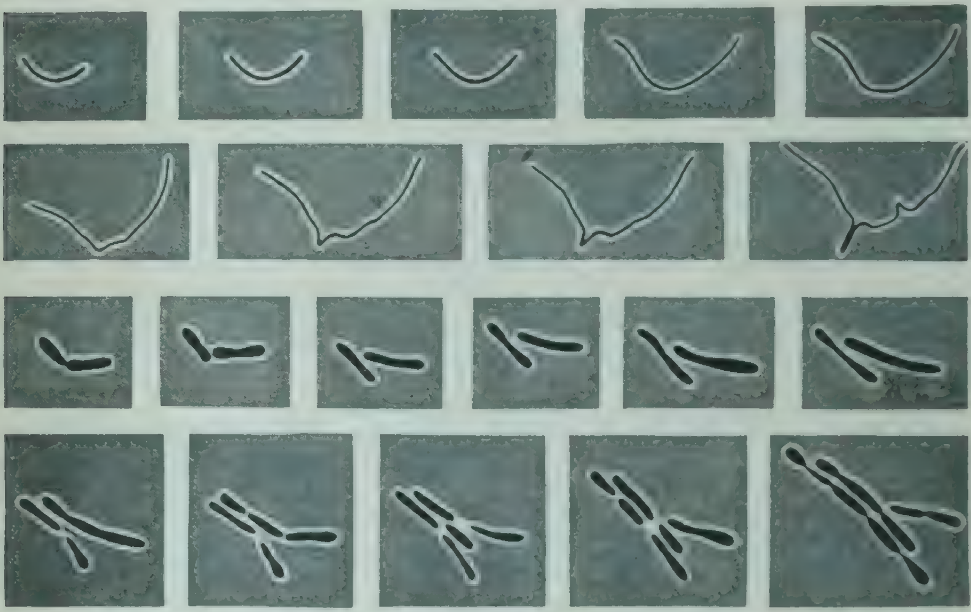

Fig. 4–2. Cell growth in rough (R) and smooth (S) cultures of a species of *Bacillus*. Drawings of successive growth stages of a cell. *Above*. Rough culture, showing tendency of cells to remain in a chain, and to loop. *Below*. Smooth culture, showing tendency of cells to separate and slip past each other. (Adapted from Lasseur.)

Some of the filamentous bacteria (trichobacteria) show a much more complex morphology, producing long filaments which except for their small diameter resemble somewhat the *hyphae* of the fungi. Some kinds show branching, others are unbranched.

Variability of Bacterial Morphology. A single kind of bacterium may show in differing environments distinctly different morphologies.

Under certain conditions, perhaps not favorable for growth, bacteria may show abnormal or unusual shapes. These are sometimes termed *involution* forms. Certain species of bacteria show this characteristic much more readily than others. Figure 4–3 illustrates one of the commoner types of involution forms. Several theories have been proposed to account for the presence of these so-called involution, or unusual, or degenerate, forms of bacteria in a culture. Some have claimed that they are due to abnormalities produced in the cells by the attacks of exceedingly tiny parasites. Others have concluded that they are normal growth forms and occur at regular intervals in the "form cycle," or growth cycle, of an organism. Many others contend that these unusual forms are the result of specific influence of the environment and appear only when conditions are not favorable. It is probable that there is some truth in all these statements, that no one explanation will cover all cases. Very probably, as will be noted later, the attacks of bacteriophage may bring about changes in morphology. Not improbably certain of the bacteria

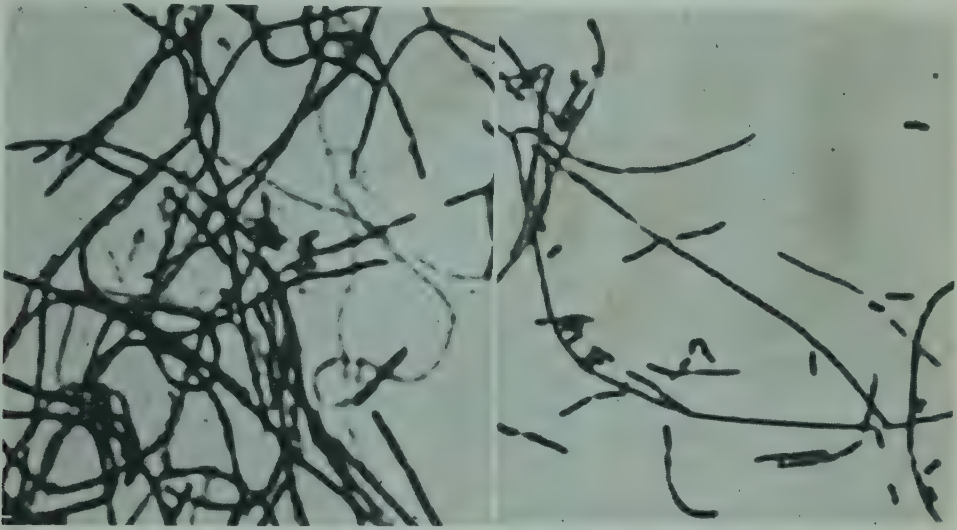

Fig. 4–3. Involution forms. Photomicrograph of a species of *Salmonella* which has been subjected to an unfavorable environment through the presence of penicillin. A few normal short rods are present. Most of the cells have developed into long filaments, in some cases showing some tendency to the formation of branches. In some of the filaments a considerable amount of degeneration has taken place, the stain is taken up lightly, there is little evidence of the presence of protoplasm. Such cells are sometimes termed ghost cells.

show form cycles in their growth in a fashion quite analogous to some of the molds. There is no doubt that changes in environment may produce profound changes in the shape and size of the bacterial cell.

Three distinct types of morphological changes are to be differentiated (Thornton, 1930). In some cases an entirely distinct type of cell originates suddenly and may be grown more or less indefinitely without reverting to the original type. Such, for example, is the change from "smooth" to "rough" colonies discussed later. In other cases the morphology is modified as the culture grows older, apparently, in response to the changes taking place in the environment. In a third type of change there is a modification of the cells correlated with some method of reproduction, such as spore production, cyst formation, gonidia production, or development of swarm cells.

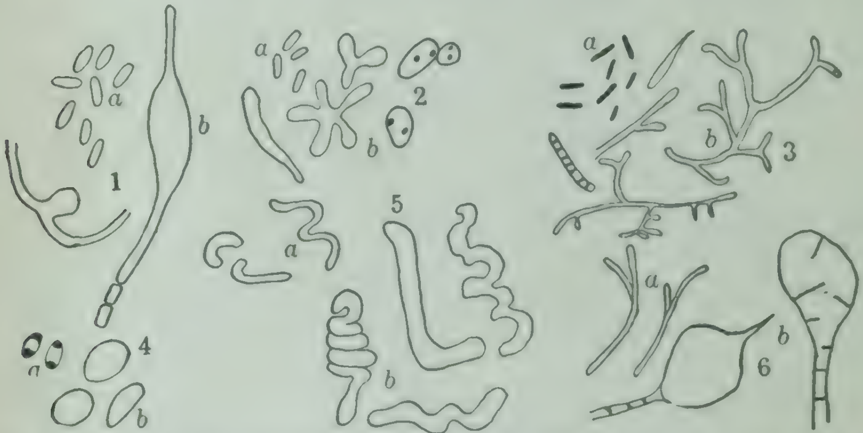

Fig. 4–4. Normal and involution forms of the cells of certain bacteria. *a*. Normal cells. *b*. Involution forms. 1. *Acetobacter acetosum* (adapted from Henneberg). 2. *Rhizobium leguminosarum*. 3. *Mycobacterium tuberculosis*. 4. *Pasteurella pestis*. 5. *Lactobacillus delbrueckii* (adapted from Henneberg). 6. *Actinomyces sp.*

Structure of the Bacterial Cell. Earlier students of bacteria employed with some success the methods which had been developed to study the cell structures of higher plants and animals. Simple methods of staining and observation were adequate for many studies relating to the economic significance of the organisms. However, the minute size of most bacterial cells has interfered with getting at details, for many purposes the resolving power of the microscope has proved inadequate. In recent years the electron microscope with its much greater power of resolution has added materially to our knowledge.

The bacterial cell is a true cell of the same general type as is to be found in other plants and animals. It probably is somewhat more primitive in its organization than are the cells of higher forms, but there is ample evidence of a considerable degree of complexity. The cell is surrounded by a cell wall, which in turn may be enclosed in a slimy or gelatinous layer called a *capsule*. It contains the living material or *protoplasm* in which there may be embedded various granules or *cell inclusions*. Recent evidence seems to justify the conclusion that the protoplasm is in turn made up of a *nucleus* and *cytoplasm*. There may also be external organs of locomotion called whips or *flagella* (singular *flagellum*).

Cell Wall of Bacteria. The wall of the bacterial cell is usually a relatively firm membrane surrounding the living cell contents. Its existence has been adequately confirmed by the use of differential staining techniques and more recently by the use of the electron microscope. Bacteria of certain species may be caused to burst and the protoplasm extruded, leaving behind the empty cell wall. "Shadow" or "ghost" cells are frequently observed when cell contents have been dissolved away, the "shadow" consisting primarily of the cell wall. The contained protoplasm in some bacterial cells may be caused to shrink away from the cell wall making the latter more easily visible. When an endospore forms within a bacterial cell, the cell wall may remain evident as a sac. Intense sound waves of very high pitch (supersonic waves) may break cells open allowing the protoplasm to disintegrate and escape, leaving the empty cell walls as "ghosts" showing clearly the lines of rupture. The electron microscope has made these essential facts even more clear. Mudd and Anderson have stated that "A cell wall,[8] structurally distinct from the inner protoplasm, can be detected in electron pictures of each of the kinds of bacteria thus far adequately studied: this includes most microorganisms pathogenic for man: cocci and rod forms, both gram-positive and gram-negative and spiral forms."

Knaysi (1944) has shown quite clearly that in most "stained mounts" examined with the light microscope the cell wall itself is unstained, and it is only when mordants and special staining technique are used that the cell wall becomes clearly visible.

The chemical composition of the bacterial cell wall is not adequately known. Color reactions (such as those obtained by use of chloriodide of zinc) have shown that in but very few instances is the cell wall made of cellulose as in many of the higher plants. In some

[8] *J.A.M.A.*, **126**, 562 (1944).

cases it is found to contain nitrogen, perhaps resembling the mucins which upon hydrolysis break down into carbohydrate and protein. In other cases, when hydrolyzed, the cell wall is found to consist essentially of carbohydrate "building blocks," including uronic acids, and to be either complex carbohydrates or related to the pectins.

The cell wall of bacteria may be regarded as a protective membrane. All the foodstuffs which enter the cell must diffuse through the wall; it acts as a relatively permeable membrane. As noted presently it may become considerably thickened, be encrusted with iron hyroxide or other materials, or it may differentiate on the exterior into a capsular substance which swells greatly in water. In most bacterial cells the wall is relatively stiff, in some it is thin and pliant, making possible bending movements of the cell.

Cell Contents of Bacteria. The protoplasm or living material usually occupies most of the interior of the bacterial cell. Generally in living cells the protoplasm is differentiated into a structure termed the *nucleus* (see below) and the *cytoplasm* constituting the remainder. The portion of the protoplasm immediately adjacent to the cell wall is differentiated somewhat as a layer (as is true generally in plant cells) known as *the cytoplasmic* or *plasma membrane*. This acts as a true semipermeable membrane, i.e., it is so constituted that some substances in solution will pass through it, others will not. In general it determines what may diffuse into and from the cell. The presence of this functional cytoplasmic membrane may be demonstrated by placing certain bacterial cells in concentrated solutions (such as those of salt or sugar), water passes from the interior of the cell, the protoplasm shrinks, and in some cases separates from the cell wall. The cytoplasmic membrane constitutes the outer layer of this mass. A cell showing such shrunken protoplasm is said to be *plasmolyzed*, the cell has undergone *plasmolysis*. These terms are not particularly appropriate, however, for the literal meaning of plasmolysis is "dissolving plasma" which is not at all the same as shrinking of the protoplasm. Careful study of bacterial cells shows however that they are much less sensitive to changes in the solution concentration of their environment than are the cells of most other plants or animals and show evidence of plasmolysis less frequently.

There is not complete agreement among bacteriologists that the bacterial cell contains a nucleus, at least one comparable with that found in other plant cells. A typical plant nucleus is a more or less spherical body lying in the cytoplasm of the cell and containing dense granules or threads which are readily stained intensely by certain dyes and are called in consequence *chromatin*.[9] When the cell divides, the chromatin organizes into definite rods or masses which are termed *chromosomes*. The delicate membrane around the nucleus disappears, each chromosome divides, and one half goes to each end of the cell, where a new nucleus is organized.[10]

Does the bacterial cell contain a nucleus similar to that just described as characteristic of higher plants? Many points of view are to be found among those who have studied the subject. Opinions vary from belief that bacteria have nothing corresponding to a nucleus, that the protoplasm is a nucleus without surrounding cytoplasm, that the nucleus consists of tiny granules of chromatin scattered through the protoplasm, that a typical nucleus is present and that the nucleus is represented by one or more naked chromosomes.

However, evidence has rapidly accumulated in recent years, indeed seems reasonably adequate, that the bacterial cell does not differ materially from the cells of many of the fungi and does possess a structure which functions as a simple nucleus. One reason for the slowness of recognition of the bacterial nucleus has been the fact that when most bacterial cells are stained with *basic dyes*[11] the entire mass of protoplasm usually stains heavily and quite uniformly. This is in contrast to the behavior of most other cells in which the nucleus stains much more intensely than does the cytoplasm. This led students in some cases to infer that perhaps the entire interior of the cell consisted of a nucleus, and there is little or no cytoplasm. Others believed that inasmuch as chemical analyses showed the presence of materials characteristic of chromatin, the nuclear material was scattered throughout the protoplasm.

Better methods of staining were developed. Tests were discovered which were even more accurately diagnostic than the basic stains, particularly the Feulgen[12] reaction. The use of these tests showed clearly the presence of chromatin. An adaptation of the methods used in staining protozoa revealed consistently the presence of nu-

[9] From the Greek noun *chromus* meaning color or stain.

[10] The student should consult a standard textbook on biology if not familiar with the usual process of cell division in plants or animals. He should have some knowledge of the hereditary mechanism.

[11] See chapter on staining methods for differentiation of acid and basic dyes.

[12] One of the constituents of nuclei or better the chromosomes of cells in so far as they have been studied is a complex type of compound called desoxyribonucleic acid. The Feulgen test is used for the recognition of this material. When properly applied, the Feulgen color reaction is perhaps the best criterion for the presence of nuclear substance.

cleus-like structures in many bacteria. It was then found that by the use of a substance such as hydrochloric acid the tendency for the cytoplasm to stain intensively was eliminated, but without much change in the staining power of nuclear chromatin. When cells thus treated are properly stained, they quite regularly show structures which resemble nuclei, or more accurately, chromosomes.[13]

The work of Robinow has been particularly significant in making probable the existence in certain bacteria at least of a chromosome-like body. In working with the members of the bacterial genus *Bacillus*, he found each cell to contain a dumbbell-shaped body which functioned quite as a chromosome. Similar chromosomes were later found in the cells of bacteria of many species belonging to many genera. This "chromosome" divides longitudinally, before each division of the cell. The division of the bacterial cell does not always follow immediately the division of the chromosome, giving rise to a series of the dumbbell-shaped bodies, usually in pairs, in an elongated cell. Such cells would then resemble the multinucleate cells often observed in fungi.

The possession of a chromosome structure would make possible a heredity mechanism in bacteria comparable to that of higher forms. Geneticists are in general agreed that hereditary characters are in part determined by so-called genes which are arranged as a chain in the chromosomes. When a chromosome increases in size and splits lengthwise, each gene divides, and each daughter chromosome possesses a replica of the gene chain of the mother chromosome. Bacterial cells transmit to their daughter cells the characteristics of the mother cell with great fidelity. There are exceptions to the rule among the bacteria exactly as with higher plants. It would seem logical to assume that the mechanism of transmission of characters from one cell generation to the next in bacteria is similar to that observed for other forms of life.

Granules and cell inclusions of various kinds are sometimes observed in bacteria, among them *metachromatic granules*, granules of *glycogen*, *sulfur granules*, and *oil* or *fat globules*.

Metachromatic granules are so named because they take up the basic aniline dyes as does chromatin. They are highly refractive and hence easily observed. They are common in such organisms as the diphtheria bacillus and in certain lactic acid bacteria. Their presence or absence may be significant in disease diagnosis and in differentiation of bacterial species.

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[13] For a much more detailed but very readable and authoritative discussion of the whole problem of bacterial cell structure, the student should read Dubos, *The Bacterial Cell*, Harvard University Press, 1945, and its contained Addendum by Robinow.

Glycogen granules are produced by some bacteria. Glycogen is related to starch in that when it is hydrolyzed it breaks down into dextrose, but it is not identical in structure. It turns brown or reddish brown and not blue when treated with a solution of iodine. Glycogen is probably reserve food.

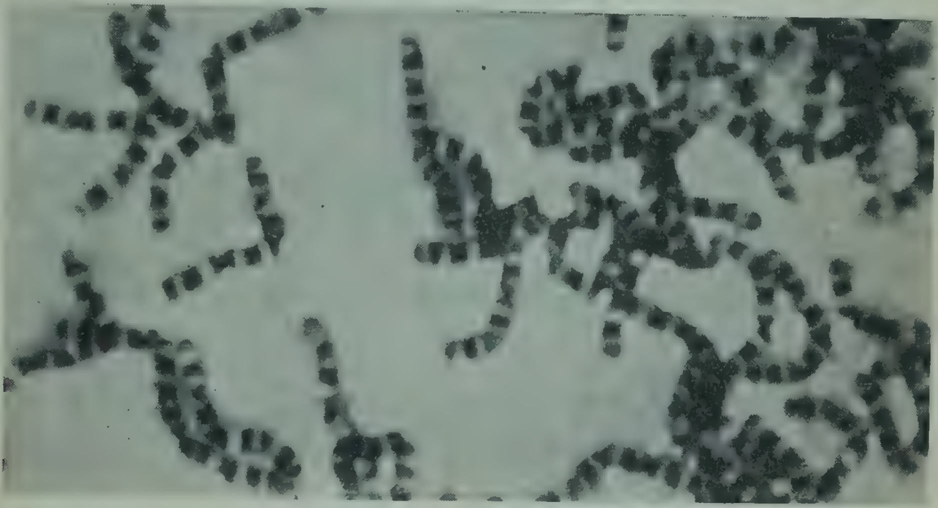

Fig. 4–5. Cell inclusions in bacteria. Photomicrograph. Note the regular banding of the cell. The bands stain intensely with aniline dyes. These bands may represent nuclear material within the cell. Probably a member of the genus *Simonsiella*. From the surface of a decaying fungus.

Sulfur granules are deposited in the cells of certain bacteria which grow in water containing hydrogen sulfide, such as that coming from sulfur springs, or in the effluent of septic tanks containing certain types of sewage. That the granules are truly sulfur may be shown by drying a mass of the organisms on a glass slide, then adding a drop of carbon disulfide, and allowing the latter to evaporate. The sulfur which has dissolved in the carbon disulfide will separate as easily recognizable crystals.

Oil and fat globules are occasionally produced by some bacteria. They may be identified by the use of certain fat-soluble dyes and by solubility. They are probably to be regarded as reserve food.

Capsules and Sheaths. A capsule is a much thickened gelatinous outer portion or covering of the cell walls of certain bacteria. Probably most bacteria possess a very thin layer of capsular material, but only

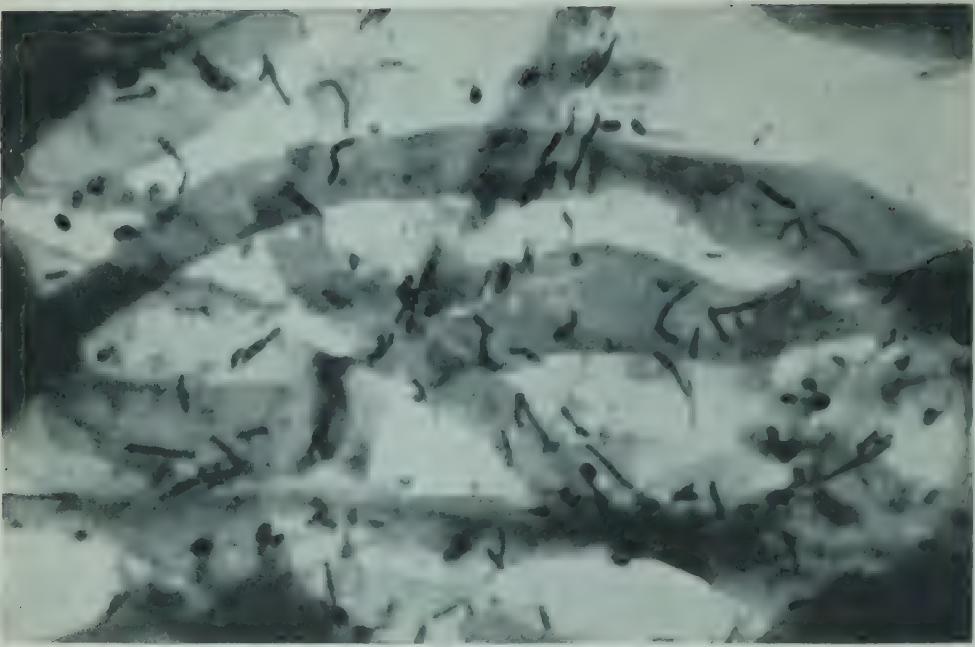

Fig. 4–6. Relative size of bacteria and fungus hyphae. Photomicrograph of a stained preparation of a mixture of bacteria and the hyphae of a fungus (*Pleurotus*). Note that the bacteria are relatively very much more slender than the hyphae.

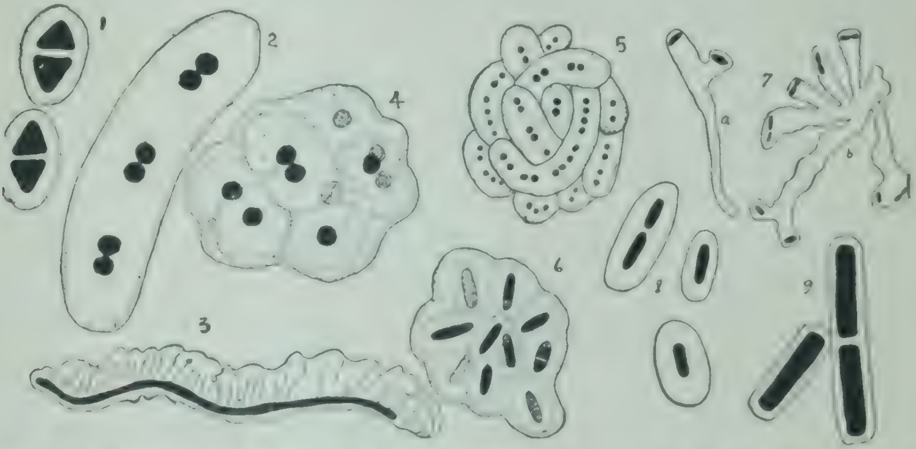

Fig. 4–7. Diagrams of some of the capsulated bacteria. 1. *Diplococcus pneumoniae*. 2, 4, 5. *Leuconostoc mesenteroides* (adapted from Lafar). 3. *Lactobacillus vermiformis* showing the asymmetrical capsular material (adapted from Ward). 6. An unidentified organism showing rods embedded in a zoogloeal mass, from sewage. 7. *Bacterium pediculatum*, showing the unilateral development of the capsular material (adapted from Koch and Hosaeus). 8. A rod-shaped organism from slimy milk. 9. *Bacillus anthracis* from the blood.

in certain species or only under certain conditions does it become conspicuous. The capsule may remain somewhat firm and gelatinous, or it may partially or wholly dissolve, rendering solutions in which the bacteria are grown slimy or gluey. Such, for example, is the origin of slimy milk and cream. The capsular material produced is characteristic of the organism producing it; it may or may not be nitrogenous. A substance closely resembling the mucin found in mucus has been identified from some species. Others upon suitable digestion break down into either hexose or pentose sugars. In some cases uronic acids have been detected as a product of hydrolysis, indicating a relationship to the pectins of the higher plants. Such seems to be the nature of some of the bacterial gums. The individual cell may be separately capsulated, or a chain or group of cells may be contained in a common capsule, or the bacterial cells may be embedded in a more or less formless mass of gelatin. Such a mass of organisms with surrounding gelatinoid material is sometimes termed a *zoogloea,* a name meaning *animal glue* which has persisted since the early days of bacteriology when such masses observed in stagnant water were thought to be composed of animal forms.

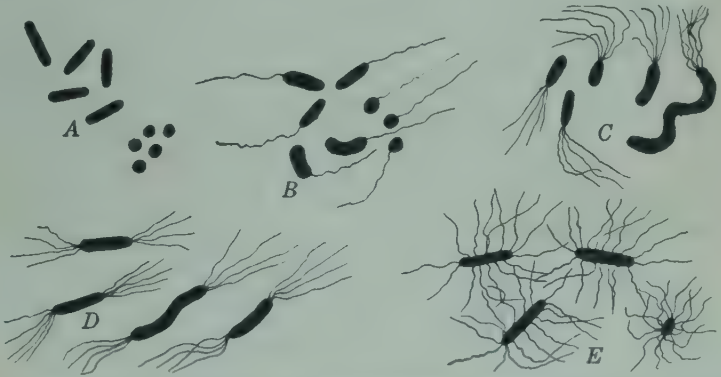

Fig. 4–8. Flagella of bacteria. A diagrammatic representation of the manner in which the flagella may be distributed on the surface of the organisms. A. Rods and spheres without flagella (non-motile, atrichous, atrichic). B. Cells each with a single flagellum (monotrichous or monotrichic). C. Cells each with a cluster of flagella at one pole (lophotrichous or lophotrichic). D. Cells with clusters of flagella at each end of the cell (amphitrichous or amphitrichic). E. Cells with flagella on all sides (peritrichous or peritrichic).

One interesting group, the *stalked bacteria,* produced the capsular substance asymmetrically, that is, in much greater abundance upon one side of the organism. The result is a gelatinous stalk which may branch as the bacteria divide. In some cases one end of the stalk is attached to a solid surface, constituting a holdfast.

A few bacteria have been described which grow in chains within a *sheath*, a firm membrane formed about the entire filament. In some cases the sheath may be coated with a deposit of iron compounds, as in the iron bacteria.

Organs and Methods of Locomotion. Many bacteria have the power of independent movement. Some kinds are able to swim or to move about (are *motile*) throughout the life of the cell. Others are motile only during certain stages of cell development, others are always *non-motile*. Several methods of moving about are to be found among the bacteria. Most commonly bacterial motility is due to the presence on the cells of one or more slender, hairlike appendages termed *flagella*[14] or whips. The flagella vibrate actively, propelling the organism. The flagella are in general so slender and in consequence so difficult to observe that there has been some doubt as to their attachment and origin. Some have thought them to be outgrowths of the gelatinous layer outside of the cell wall, but evidence secured by the electron microscope makes reasonably certain that they are connected through the cell wall to the protoplasm of the interior. There is some evidence that each may terminate in a basal granule in the protoplasm as do the similar organs of motion among the protozoa. The flagella are usually so slender and fragile that special techniques are needed to observe them. It is possible by means of mordants and dyes to make them visible in suitably stained preparations. Their form and attachment may be shown particularly well by the electron microscope. As noted above, not all bacteria are motile by means of flagella. Relatively few species of cocci, many of the bacilli, and most of the spirilla are motile.

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[14] The beginning student will do well to recognize that many words of Latin origin are used in bacteriology (as in most biological sciences) and that they in the English frequently follow the Latin in formation of the plural. Most (not all) Latin nouns ending in *-us* (as bacillus) form the plural by changing the ending to *-i* (as bacilli), most ending in *-a* (as sarcina) change to *-ae* (as sarcinae), and those ending in *-um* (as spirillum) change to *-a* (as spirilla). Words which end in *-um* are apparently particularly troublesome. The most common error is the use of a plural noun with a singular verb. One of the signs of scientific illiteracy is to write "This data is" for "These data are," and particularly in bacteriology "This bacteria is" for "This bacterium is." The following singulars and plurals should be noted as most commonly incorrectly used: *datum—data; flagellum—flagella; bacterium—bacteria; cilium—cilia; spirillum—spirilla; septum—septa; stratum—strata; mycelium—mycelia; conidium—conidia; sporangium—sporangia; clostridium—clostridia;* and *medium—media.* In some cases a second plural form (as *mediums*) is also recognized.

Observation of the number and location of the flagella on the organism permits a separation of bacteria into several groups. An organism with a single flagellum at one end of the cell (*polar*) is *monotrichous;* one with a cluster of two or more flagella at one end is *lophotrichous;* one with a cluster at each pole *amphitrichous;* and one with flagella on all sides *peritrichous*.[15] Some authorities do not differentiate between lophotrichous and amphitrichous bacteria, since the latter are merely a growth phase of the former.

Two groups of bacteria contain species which are motile but do not possess flagella. In one of these groups, the spirochetes, the cells are very slender and relatively long. They seem to swim in much the same fashion as a snake or an eel, by a sinuous bending of the cell body, that is, they are flexuous. It is this type of motility which has been cited by some observers as one evidence of their protozoan (animal) affiliations.

In the second of these motile non-flagellate groups of bacteria the cells glide or move along the surface of a solid object. The slime bacteria (myxobacteria) have this type of motility, as do some of the sulfur bacteria and certain kinds of algae. The mechanism of this peculiar motion has not been satisfactorily explained. Frequently an organism moving in this fashion is observed to leave a trail of slime behind, and it has been conjectured that the cell is pushed along by the extrusion or secretion of the slime at certain points on its surface.

Reproduction of Bacteria. Bacterial cells may either be in an active growing stage (vegetative state) or in the form of resting cells. Actively growing bacterial cells may increase in numbers in several different ways. Most commonly they multiply by splitting crosswise, that is, by transverse (never longitudinal) fission. This method of multiplication is so common and so characteristic of the bacteria that they have been given the name of *Schizomycetes* (fission fungi). Bacteria are also known which may increase by producing buds from the surface of the cell; these may break away and grow into mature cells. Some bacteria have been described as multiplying by segmentation of the cell contents, followed by release of the small organisms which develop into cells of normal size.

Some bacteria produce specially resistant cells fitted to *reproduce*

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[15] Some writers use the alternative adjectival forms, *monotrichic, lophotrichic,* and *peritrichic.*

NOLOGICAL RESEAR

the species after a period unfavorable for growth, but in such cases no actual multiplication necessarily takes place. These cells are termed *spores*.

The variety of methods of cell reproduction among bacteria and the morphological variation of the cells in older cultures have led some investigators to believe that bacteria may have complex life cycles. The alternation of the rapid growth phase (vegetative reproduction) with production of resting cells (spore production) is evidence of at least a simple cycle. Some evidence has been brought forward that there may be some type of sexual reproduction among bacteria; however, it is not as yet conclusive, and bacteria are usually assumed to be non-sexual.

Vegetative Multiplication and Reproduction. As indicated above, the most common method of bacterial multiplication is by transverse fission. The actively growing cell produces a transverse wall which splits, and the two daughter cells are separated. The cells may remain united by capsular material. The plane of this division is always at right angles to the longest axis of the cell. In the more usual reproduction by fission, the nucleus (in those forms in which it has been demonstrated) divides to form two nuclei which separate, a wall forms between the two halves of the cell, and finally the cells may split apart. In some cases the nucleus may divide more rapidly than walls form, giving rise to elongated cells possessing several nuclei. In some types of bacteria it is not improbable that the normal condition is that of cells containing several nuclei (multinucleate cells) such as are formed in certain molds and algae.

The division of cells may occur with considerable rapidity, a short time only being required for the organism to grow to full size and split into two cells. Many species of bacteria may reach maturity and divide in half an hour; in some cases, even in eighteen to twenty minutes. Under favorable conditions of temperature and food supply this rate may be maintained for a considerable period; but the gradual accumulation of the metabolic products or the using up of the available food finally diminishes this rate. An organism multiplying in this geometric ratio in the course of two days would have a progeny represented by a number having twenty-nine figures. In a few days, such multiplication would result in the formation of a mass of bacteria equal in bulk to the earth itself. Conditions, of course, are never long favorable for such indefinite increase. This rapidity of multiplication is of the greatest economic importance, however, for it enables bacterial cells, in spite of the slight bulk of a single organism, to produce very considerable chemical changes in a short time, as in fermentation. Milk, for example, with an initial seeding of a very few bacteria capable of producing lactic acid, if allowed to stand in a warm place, will sour within a few hours.

There are to be found in the literature of bacteriology numerous examples of bacterial multiplication by methods other than fission. Some forms are described as multiplying by budding, others by a process of internal cell fragmentation to form reproductive bodies termed *gonidia*. One of the most clearly described cases of reproduction by gonidia is that of the bacteria which produce nodules on the roots of leguminous plants. The motile cells elongate, and their contents become granular, several granules eventually occupy a cell. The cell wall then ruptures and the granules (gonidia) emerge as "swarming" or "swarm" cells.

Spore Production. A *spore* is a cell (sometimes a group of cells) set apart for reproduction, usually differing somewhat in shape, size, or in other characters from the vegetative or normal growing cell from which it develops. Spores are more resistant to unfavorable environment such as drying than the other cells of the organism. They are of many shapes and sizes and are given various names based upon their morphology. They are produced by some bacteria, by most yeasts, by most molds, and by some protozoa.

Although spores are frequently more resistant to unfavorable environments than are the cells which produce them, it must not be concluded that the cells await an unfavorable environment before spore production (sporulation) occurs. Just what causes a bacterial cell to cease vegetative multiplication and produce a spore is not entirely clear for most species. Undoubtedly the immediate cause is some stimulus coming from the environment. The drying of the medium, the presence of certain salts, the exhaustion of the available food supply, the presence of products of growth, a favorable degree of acidity in the medium: these and many other environmental factors have been described as stimulating spore production.

The spore, produced by certain kinds of bacteria is known as an *endospore* because it is always formed within the bacterial cell. Not all bacteria form endospores. Most of the cocci do not. Many of the bacilli and a few spirilla are spore forming. The development of an endospore in a bacterial cell is first indicated by the appearance within the cell of highly refractile granules. These tend to collect at one point in the cell and unite to form a larger globule. This increases in size and density and finally surrounds itself with a firm membrane or spore wall. The spore seems to be formed by the contraction of a part of the protoplasm with extrusion of water. A cell containing a spore is sometimes termed a *sporangium*. Usually only a single spore develops within a bacterial cell; occasionally two are found, one at either end. The spore is usually ellipsoidal or oval in shape, rarely spherical.

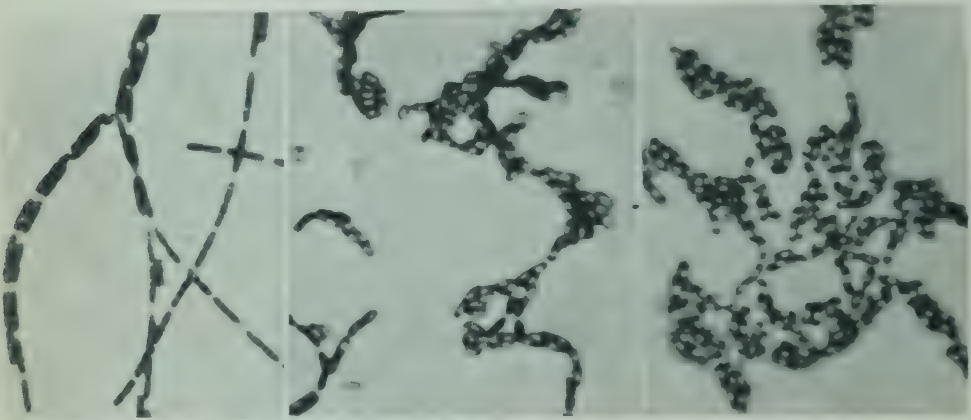

Fig. 4–9. Endospore production in a species of *Bacillus*. Microphotographs. A. Chains of rods with protoplasm stained, but not the cell walls. Note the occurrence in a few of the cells of a tiny clear or unstained area, the first evidence of the formation of an endospore. B. Late stage in spore formation. There is some tendency for the chains of cells to break apart, the clear areas in the cells are the spores. In some case the spores have broken out of the old cell wall or sporangium and are free. C. Another view of rods forming spores. Note that in this case the cells (sporangia) show deep staining at the ends.

The sequence of internal cell changes in spore formation has been worked out by modern cytological technique for few bacteria. The minuteness of the cells makes accurate observations of this sequence extremely difficult. Thus far the use of higher magnifications made possible by the electron microscope has not proved very significant. Studies of *Bacillus subtilis* have led some observers to conclude that when the cells cease the usual vegetative divisions, the nucleus (with its single chromosome) divides, and the two resultant daughter chromosomes remain, or draw, together to form a nucleus with two chromosomes. According to this theory, two successive divisions give rise to four (perhaps sometimes three) nuclei, each with a single chromosome. Three of these nuclei degenerate and disappear. The remaining nucleus is the center around which the endospore develops. As thus explained, spore formation resembles that of some of the fungi, involving a simple sexual cycle. But the evidence is not conclusive.

The position of the endospore within the cell and its diameter relative to that of the cell are of considerable assistance in the separation and identification of certain species of bacteria.

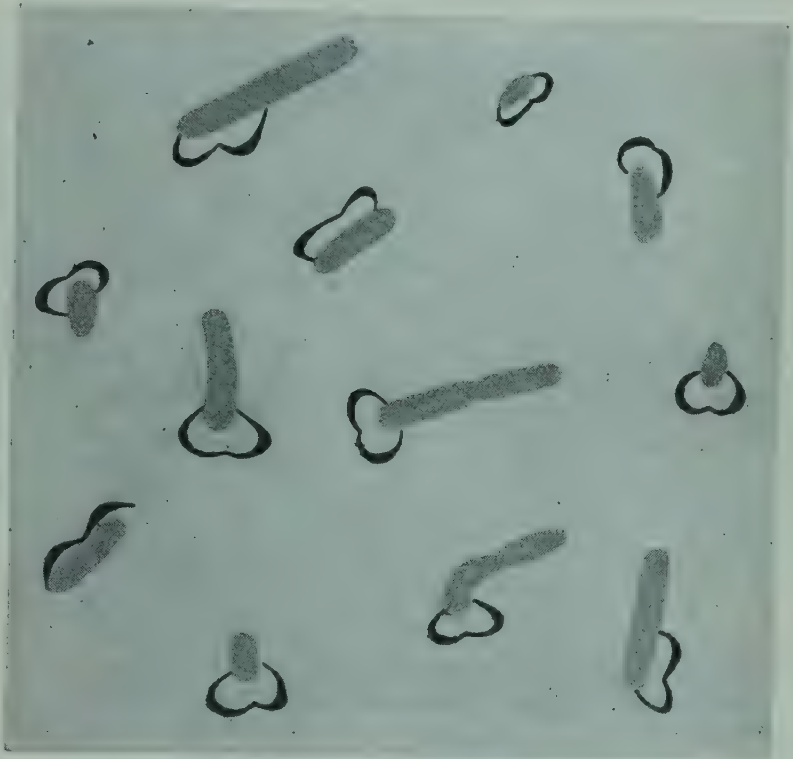

Fig. 4–10. Germination of the spores of *Bacillus mesentericus*. Note that the sporangium usually breaks open on one side, and the vegetative cell enlarges and frees itself. (Adapted from Lasseur and Benoit.)

Spores may be either *equatorial* or polar; that is, situated at the center or near the end of the cell. The spore may have a diameter less than that of the mother cell, in which case the shape of the cell may or may not be altered, or it may be greater and cause a distortion in shape. An equatorial spore resulting in the enlargement of the cell causes the formation of a spindle-shaped sporangium, usually termed a *clostridium*. Such a spore at the pole gives a drumstick type of sporangium (a *plectridium*), such as is found in the organism causing lockjaw or tetanus.

At maturity, the old cell or sporangial wall generally disintegrates, liberating the endospore. In a few species the wall is persistent. A spore may germinate when it encounters favorable conditions for growth. Germination may be accomplished by a swelling of the spore contents and a stretching of the membrane or by its rupture. If the latter, the break may occur at one or both poles, or at one side. The spore contents emerge as a vegetative cell which begins to grow and multiply. Spore-producing bacteria are sometimes differentiated into those whose spores show polar (terminal) germination, those which show equatorial germination, and those which stretch the membrane without rupturing. Each species shows a high degree of constancy in the mode of spore germination; this characteristic is therefore used in differentiation and identification of various kinds of bacteria.

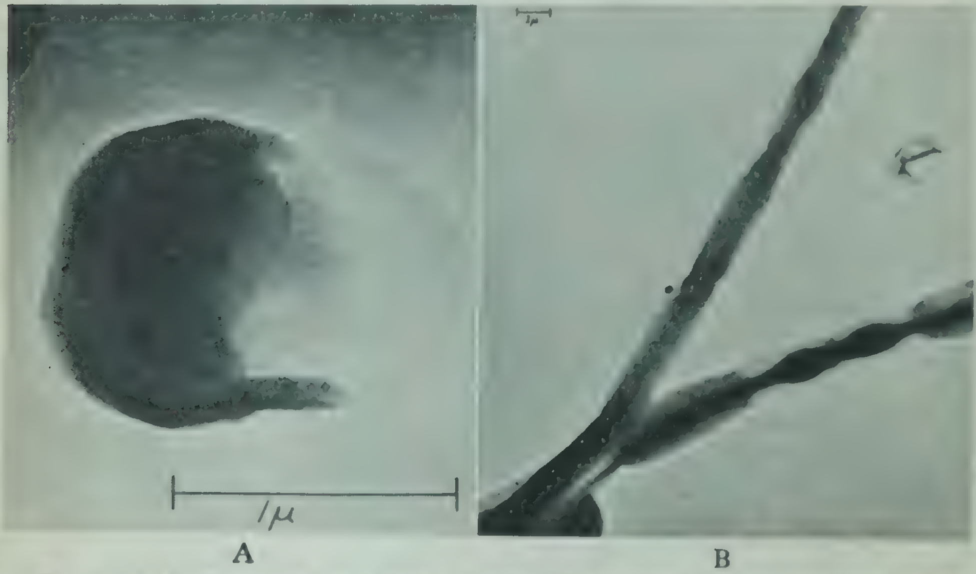

Fig. 4–11. A. Germinating spore of *Bacillus mycoides*. Electronograph. The spore was placed on nutrient agar at 35°C., and examined after two hours. The ruptured double layer of the spore wall is readily seen, with the much less dense young vegetative cell growing through the opening. (By courtesy of Georges Knaysi, R. F. Baker, and James Hillier, and the *Journal of Bacteriology*.) B. Electronograph of young spores in chain of cells of *Bacillus anthracis*. (By courtesy of S. Mudd and T. F. Anderson and the *Journal of the American Medical Association*.)

Just what occurs within the cell is not too clearly demonstrable. One observer (Bastian), who studied *Bacillus subtilis*, concluded that spore germination involves a breaking or stretching of the spore wall, and relatively rapid division of the nucleus with growth of the cell (the increase in number of nuclei outstripping the formation of cross walls) and the splitting of the cells. This sequence may lead in some cases to the presence of as many as four, or perhaps even more, nuclei in a single cell. When growth is slower, cross walls develop, and each cell contains a single nucleus.

Since in general only a single endospore develops in a bacterial cell, sporulation in such cases cannot be regarded as a method of multiplication, but of reproduction only.

Some kinds of bacteria may produce reproductive bodies or cells that are not endospores. In some cases these apparently develop from the cell by a process of budding, in others by a process of segmentation. In the latter case, particularly, they may be extremely small, so small in fact that some authors (though probably on insufficient evidence) have believed them occasionally to be ultra-microscopic. When placed under a favorable environment they may develop into the typical bacterial cell.

Caution must be used in the interpretation of buds, gonidia, etc., as reproductive bodies on bacterial cells. In some cases they apparently do represent structures for reproduction, in other cases not. For example, it has been shown by Stapp and Zycha that when *Bacillus mycoides* is placed in a medium containing magnesium sulfate in sufficiently high concentration the cell wall bursts and the protoplasm is gradually exuded in a series of droplets which bear a superficial resemblance to spores, but are quite incapable of growth.

Not infrequently bacterial cells which do not produce typical endospores may nevertheless be found to become relatively resistant to unfavorable conditions, such as drying. For example, the cells of certain cocci may be dried, and blown about in the air for a time, and still retain the ability to grow. Such cells, which are morphologically little differentiated from the vegetative cells but are more resistant, are sometimes termed *arthrospores*.

Certain of the so-called higher or filamentous bacteria produce numbers of specialized cells or spores termed *conidia*. These are formed usually at the free tip of the filament. In some cases the tip successively pinches off reproductive cells. These spores resemble very closely those produced by certain types of molds. In fact this group of trichobacteria is probably intermediate between true bacteria and molds.

Spores are usually much more resistant to unfavorable conditions, such as drying, heat, light, and chemical agents, than are the cells which produced them. This is probably due to two factors: the greater protection afforded by the spore membrane and the fact that the protoplasm of the spore contains much less water than that of the vegetative cell. It is a well-known physiological fact that a moist cell is more easily destroyed than one which is dry. Freezing, for example, will seriously injure a moist wheat kernel, but is without material effect upon one which is dry.

Sexual Reproduction in Bacteria. Closely related to the problem of nuclear organization is that of sexual reproduction. There is reason to believe that sexual reproduction of a relatively primitive type may occur in some bacteria.[16] Such reproduction in any plant or animal consists essentially of the union of two nuclei. If the nuclei uniting are not completely identical in hereditary characteristics, that is, if they do not contain identical hereditary units or genes, there is opportunity in the divisions of the nucleus for recombinations of the genes, and consequent chance for the development of cells unlike the cells of either parent. The presence or absence of sexual reproduction is of much theoretical importance in the development of explanations of variability and heredity.

A very primitive type of sexual reproduction of one of the rod-shaped bacteria has been claimed by Bastian.

There is some evidence further of nuclear fusions among the myxobacteria preceding spore formation. Proof that two separate bacterial cells ever unite or fuse has not been established, though there is some circumstantial evidence that this may occur. Such fusions are common between cells of fungi or between yeast cells. Differentiation of sex cells (formation of gametes) has not been satisfactorily demonstrated, although some authors have found evidence that it may occur. Here again the minuteness of the cells and the difficulty of watching their growth and development have thus far led to inconclusive results.

NAMING AND CLASSIFICATION OF THE BACTERIA

Thousands of species of bacteria have been named. The index of Bergey's *Manual* lists between five and six thousand different names that have been given to bacteria and related organisms. Even after noting the existence of many synonyms, the number of species recognized is great. In all probability a very small proportion of all the species which exist have been thus far discovered or described.

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[16] The evidence for this conclusion that bacteria may show some evidence of sexuality is discussed further in Chapter 8.

How is it possible to separate and identify thousands of kinds of plants so small and so undifferentiated as are the bacteria? Among the higher plants and animals it is customary to use morphology almost exclusively for differentiation. One species of oak tree may be distinguished from another by differences in the shape of the tree, the shape of the leaves, the shape, color, size, and general appearance of the acorn and its cup. With bacteria the problem is somewhat more difficult. Practically all bacteria are simple spheres, rods, or filaments. Something besides morphology must be used to tell them apart. In general bacteria are classified both on the basis of *what they do* and of *what they look like*. Physiology of bacteria is quite as important as morphology in their differentiation. Morphology is used as far as practicable: shape; size; number and arrangement of flagella; presence or absence, and position of spores; staining characteristics; presence or absence of granules. Frequently, however, no morphological differences can be found between two bacteria which are quite certainly distinct. For example, it is quite difficult if not impossible to discover any morphological difference between the organism which causes typhoid fever and certain kinds which do not produce disease. They can be differentiated, however, quite readily in the laboratory by the way in which they grow upon culture media or by the changes which they bring about in the reaction of a medium containing sugars; their ability to produce gas and acid and certain serologic reactions are also utilized in classification.

Most of the studies of the techniques of the bacteriological laboratory are designed primarily for the purpose of learning the various physiologic, cultural, and serologic methods which may be used for the differentiation of bacteria.

Systems of Classification. Many classifications of bacteria have been proposed and used. There is in existence no list of approved names imposed by authority. A classification, furthermore, must be modified as new facts are discovered. That bacteriologists are not entirely in agreement is a troublesome fact that must be faced by the student. As noted above, the criterion of correctness of names is conformity with the rules of nomenclature. Since, however, these rules were approved in final form only in 1947, time will be required to secure, or even materially to increase, uniformity. The classification and names to be used in this text are based in general upon those used in the sixth edition of Bergey's *Manual*. Changes used herein will be occasioned by the fact that in some cases the Bergey *Manual* does not conform to the international rules and in other cases more recent studies have brought to light facts which make other changes advisable. In such cases reasons for change will be given.

In the following paragraphs an outline of the classification of bacteria is presented. Keys which will enable one to differentiate the various orders, families, and genera of the greatest economic significance have been placed in the Appendix. It should be recognized by the student that there are many genera and families which are not defined at all in this text. Even after the elimination of a large proportion of the names, he will be puzzled to know what to do with the considerable number remaining. The remainder of this chapter may well be read with care, but no attempt should be made initially to commit the names to memory. The following discussion is one to which he may turn later when puzzled by the names applied to organisms. For example, a later chapter discusses transformation by bacteria of alcohol into acetic acid, that is, to vinegar. Organisms which bring about this change belong to the genus *Acetobacter*. When this chapter is studied, it is wise to review the relationships of *Acetobacter* to the other bacteria by noting just where this genus belongs in the classification.

The Class *Schizomycetes*. Bacteria are generally grouped together as a class *Schizomycetes*.[17] In general they are one-celled plants which multiply typically by transverse fission. With the exception of one small group of species, none possesses true chlorophyll. The cells may be spherical, cylindric, comma-shaped, spiral, or filamentous, and may be united into chains or into flat or cubical aggregates.

The Orders of Bacteria. Bacteria (the class *Schizomycetes*) have been variously divided into orders.[18] The present tendency is to recognize six distinct groups or orders, i.e., (1) the true bacteria (*Eubacteriales*), (2) the ray bacteria (*Actinomycetales*), (3) the spirochetes (*Spirochaetales*), (4) the slime bacteria (*Myxobacterales*), (5) the sheathed bacteria (*Chlamydobacteriales*), and the (6) rickettsiae (*Rickettsiales*). The first order *Eubacteriales* includes those bacteria which seem to be most primitive and least differentiated. In this order are to be found the major portion of the bacteria that are of economic significance. The second order, the *Actinomycetales* or ray bacteria (or fungi), includes the bacteria which are somewhat moldlike in characteristics, in several respects perhaps intermediate between the true bacteria and the molds. The third order, the *Spirochaetales* or spirochetes, includes organisms usually relatively slender, usually spiral, which are commonly motile and swim by flexing the cell. In some respects they resemble some of the protozoa. The fourth order, the *Myxobacterales*[19] or slime bacteria are rod-shaped, move about by a gliding motion, secrete considerable amounts of slime, usually produce by association of masses of cells fruiting bodies having certain resemblances to those produced by slime molds. The fifth order *Chlamydobacteriales* includes bacteria growing characteristically in filaments and having some characteristics which seem to relate them to algae; the order contains bacteria of relatively minor economic importance. The sixth order, the *Rickettsiales* (the rickettsias or rickettsiae), includes certain organisms, usually relatively minute, which have developed a very high degree of specialization; they are known only from living cells of animals, they are in general intracellular parasites causing disease. They have certain characteristics which seem to relate them both to the protozoa and to the viruses.

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[17] From the Greek *schizo*, to split, and *myces* (plural *mycetes*), fungus. *Schizomycetes* is a plural noun.

[18] In general, as noted previously, the classification outlined in the sixth edition (1948) of Bergey's *Manual of Determinative Bacteriology* will be followed. The principal difference in the classification here given is that the viruses are considered to be sufficiently distinct to be given status as a class (*Vira*) coordinate with the bacteria and are not included in the *Schizomycetes*.

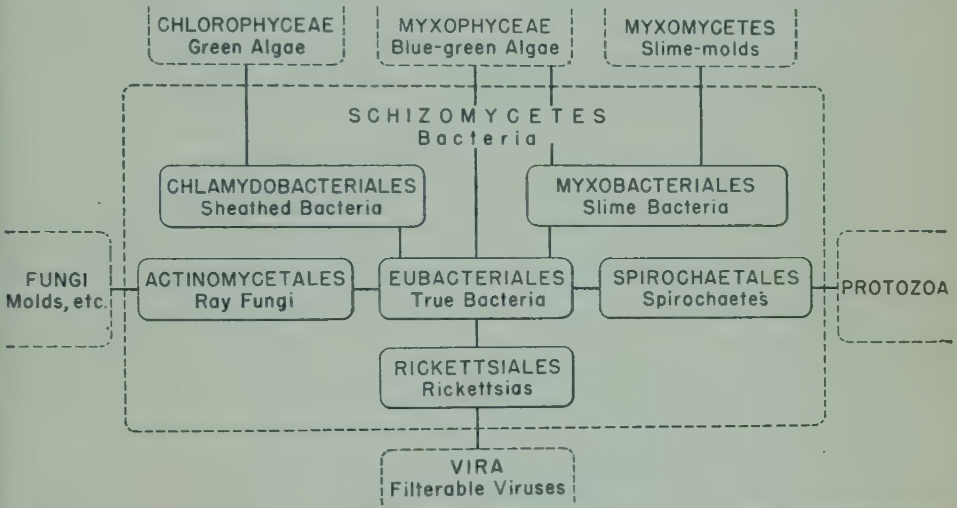

Fig. 4–12. A diagram of suggested interrelationships of the *Schizomycetes* (bacteria) and of the recognized orders of this Class. Note that the order *Eubacteriales* is centrally placed and that interrelationships held to be probable are indicated by lines. Note that the *Rickettsiales* are indicated as having some probable relationship on the one hand to the *Eubacteriales* and on the other to the *Vira*.

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[19] The correct spelling under a ruling of the Judicial Commission is *Myxobacterales* and not the older form *Myxobacteriales*.

The relationships among these orders and their possible relationships to other groups such as the protozoa, the algae, the blue-green algae, the slime molds, the molds (fungi), and the viruses are illustrated in Fig. 4–12.

The following abbreviated key may be useful in getting clearly in mind the principal characteristics used in identifying organisms belonging to the several orders.

Key to the Orders of Bacteria (Schizomycetes)

- a. Cells not minute intracellular parasites of animal cells.
  - b. Cells not enclosed in a firm sheath.
    - c. Cells not motile by gliding movement, do not produce fruiting bodies made up of masses of cells.
      - d. Cells not slender flexuous spirals.
        - e. Cells not markedly elongate nor with tendency to form branches and mycelia. 1. *Eubacteriales*
        - ee. Cells usually elongate, frequently forming branched filaments or even a mycelium. 2. *Actinomycetales*
      - dd. Cells slender spirals, motile by flexing. 3. *Spirochaetales*
    - cc. Cells show gliding movement, produce fruiting bodies made up of masses of cells. 4. *Myxobacterales*
  - bb. Cells enclosed in a firm sheath. 5. *Chlamydobacteriales*
- aa. Minute obligate intracellular parasites of animal cells 6. *Rickettsiales*

1. THE ORDER EUBACTERIALES

The True Bacteria

The *Eubacteriales* are important because the order includes a large proportion of the bacteria which are of economic significance. Here belong those bacteria which are at least specialized and differentiated morphologically, with simple spherical, rod-shaped, or spiral cells, although in physiology they show great diversity. They do not in general show any close relationships to other groups of plants such as the molds, the algae, or the protozoa.

No attempt will be made in this chapter to outline the complete classification of the families and the genera of the bacteria which are included in the *Eubacteriales*. The Appendix should be consulted for keys to the suborders, the families, and the more important genera. Here will be described only a few of the genera, more detailed descriptions will be given in those chapters devoted to discussions of their economic significance. For keys to and descriptions of all genera a manual should be consulted.

The order *Eubacteriales* has been divided into three suborders, the typical true bacteria (*Eubacteriineae*), the stalked bacteria (*Caulo-*

bacterineae), and the purple bacteria possessing pigments which function as does the leaf green (chlorophyll) of higher plants (the *Rhodobacteriineae*). Most of the bacteria to be discussed belong to the first of these suborders; it alone will be considered here.

Thirteen different families of the *Eubacteriineae* are listed in the Bergey *Manual*. One of these, the *Bacteriaceae* is a catchall for organisms insufficiently described to be classified and named properly. All the remaining twelve families contain one or more genera of significance to us. The keys to the families and to the more important genera are placed in the Appendix.

Bacillaceae. This family includes those straight rod-shaped bacteria which are capable of producing endospores. Some species are motile by means of peritrichous flagella, some are non-motile. The spores may be cylindrical, ellipsoidal, or spherical, and may be central, polar, or subpolar. In some cases the cell (sporangium) may be swollen in size, making it spindle-shaped or drumstick-shaped. Two genera are commonly recognized.

Bacillus[20] is the generic name used for those species which are able to grow in contact with air (oxygen), i.e., are aerobic. Many species have been described, some to be discussed later as having significance in fermentations or in soil or food; others as capable of producing disease such as anthrax (caused by *Bacillus anthracis*) in animals and man and others in the larvae of certain insects of economic importance.

The genus *Clostridium* includes those sporulating rods that do not grow in the presence of air (are *anaerobic*) or grow only in very low concentrations of oxygen (are *microaerophilic*). The name *Clostridium*[21] was given because some species (by no means all) produce cells which are swollen at the center when an endospore forms. Many species are very active in producing fermentations of carbohydrates, with the formation of acids (such as butyric) and frequently gas. Proteins are actively attacked by other species responsible for putrefactive changes. Some species produce poisons of various kinds which are responsible for certain diseases of man and animals such as botulism, malignant oedema, gaseous gangrene, and tetanus.

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[20] Do not confuse the common English noun *bacillus* which may be used to designate any rod-shaped organism and the generic name *Bacillus* (capitalized) which includes only certain spore-bearing rods. The Latin *bacillum* from which *Bacillus* is derived means a little staff or stick.

[21] From the Greek *closter*, a spindle, with the diminutive ending *-idium*, hence *Clostridium* = little spindle.

Pseudomonadaceae. This family includes those rod-shaped bacteria, either curved or straight, usually motile by means of polar flagella, and incapable of growing without some organic nutrients. Many genera have been described, twelve are included in Bergey (6th ed.). Of these five are of some economic significance.

Pseudomonas[22] includes a large number of species usually found in water, in soil, producing disease in plants, sometimes in animals. Usually actively motile by means of one to three polar flagella. Many species produce water-soluble and diffusible coloring substances (pigments) usually greenish, greenish-yellow or bluish, sometimes brown in color. Some produce light (are phosphorescent). Of the 148 species described in Bergey's *Manual* (6th ed.) more than half produce disease in plants.

The genus *Xanthomonas*[23] is closely related to *Pseudomonas*, with the cells motile by means of a single polar flagellum, differing in that the bacteria when grown in the laboratory are yellow in color, the pigment not being soluble in water and not diffusing. The genus is of significance as the species contained almost all produce disease in plants.

The species of *Acetobacter* are differentiated largely on the basis of lack of diffusible or yellow pigment, and by the ability of the cells to oxidize ethyl alcohol to acetic acid; they are the characteristic bacteria of vinegar. The cells of some species may be non-motile.

The genus *Vibrio* is separated on the basis of the rods being somewhat curved, not producing a soluble pigment, with cells motile by means of one to three polar flagella. Some species are pathogenic to man and animals. The best known is the *Vibrio cholerae* which is the cause of Asiatic cholera in man.

The genus *Spirillum* includes organisms of a size rather larger than those of species of other genera of this family. The cells are motile by means of a considerable tuft of polar flagella. Most species are free-living in water. There is some question as to whether this genus is sufficiently closely related to the other genera of the family to belong with them. It is sometimes placed in the distinct family *Spirillaceae*.

Azotobacteraceae. This family contains a single genus, *Azotobacter*. The cells are relatively large rods, sometimes almost spherical and somewhat yeastlike in appearance. Flagella when present are peritrichous. No endospores are formed. The cells grow only in the presence of oxygen. The species inhabit soil and are of special economic significance because of their ability to take up, fix, and utilize free nitrogen. They contribute to the development of soil fertility.

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[22] From Greek *pseudus*, false, and *monas*, a unit. *Monas* was early used as a generic name in protozoology, the meaning of *Pseudomonas* may be taken as false *Monas*.

[23] From Greek *xanthus*, yellow, and *monas*.

Micrococcaceae. This family includes those forms with spherical cells which occur in irregular masses, in regular cubical packets or in tetrads, never in chains. Usually the cells are gram-positive, if gram-negative they do not occur in pairs. Three genera are recognized.

The genus *Micrococcus* is a large one, containing many species. The cells occur in irregular masses or separately, not in chains or regular groupings. Some produce colors (pigments). The cells are usually non-motile though there is one species with motile cells. Spores are not produced. The members of this genus are common in water and some of them are pathogenic, causing inflammation and pus production in the body, others when they multiply in foods are responsible for certain types of food poisoning. Frequently certain of the pathogenic species and those living on or in the body are placed in a separate genus *Staphylococcus.* The latter term is more frequently used in the literature of medical bacteriology than is *Micrococcus.*

The genus *Gaffkya* is small, including spherical organisms which grow in the animal body and under certain conditions in the laboratory in fours (tetrads).

Sarcina includes certain interesting bacteria of soil and water in which the spherical cells when under suitable conditions form regular cubes. The cells apparently divide successively in each of three perpendicular planes and remain attached. None is of special economic importance.

Neisseriaceae. This family includes two genera characterized by having the gram-negative cells in pairs or in masses. All species described are parasitic in the body of man or animals. Usually the adjacent sides of paired cells are flattened; the two cells are often compared to paired coffee beans.

The genus *Neisseria* includes about a dozen species. The cells are normally in pairs. Some species are highly pathogenic producing such diseases as one type of meningitis and gonorrhea. Most species grow in the presence of air. The cells are usually larger than those of species of the following genus, about 1.0 μ in diameter.

Veillonella includes small (0.5 μ) spherical gram-negative organisms, parasitic in man and animals; with cells rarely paired but in irregular masses, and growing only in the absence of oxygen. Rarely are the species of the genus pathogenic.

Lactobacteriaceae. The genera of this family are grouped together primarily on the basis of their physiology, they show wide variations in morphology from cocci to long rods. All are alike in being gram-positive, and in suitable environment they ferment carbohydrates with production of acid. They are non-motile and do not produce spores. The family has two tribes separated on the basis of cell shape. The tribe *Streptococceae* is characterized by spherical cells occurring in chains or pairs. In the tribe *Lactobacilleae* the cells are rather definitely rod-shaped, sometimes relatively long. The tribe *Streptococceae* includes three genera of considerable importance, *Diplococcus, Streptococcus* and *Leuconostoc.*

Diplococcus is a small genus, consisting of species of organisms generally parasitic in man or animals. The cells are typically in pairs, with the sides in contact flattened, and with the opposite end somewhat elongated and pointed. The most important species is the *Diplococcus pneumoniae,* the commonest cause of lobar pneumonia.

Streptococcus is a relatively large genus, of which scores of species have been named. However, many of them were imperfectly described, or represented minor variants. Bergey's *Manual* (6th ed.) recognizes 24 species. Most are found parasitic in man or animals or growing in milk. The spherical cells usually occur in chains. Several species are of economic importance. Some as *Streptococcus pyogenes* cause disease, some as *Streptococcus lactis* are significant in fermentation of milk and in cheese production.

The third genus *Leuconostoc*[24] includes few species. It is differentiated from *Streptococcus* in ability to produce gas (CO_2) in fermentation of sugars. Organisms of this group are of some importance in fermentations, one species *Leuconostoc mesenteroides* produces slimy or gummy fermentation of cane sugar syrups.

The tribe *Lactobacilleae* includes four genera. The cells are generally rod-shaped, frequently quite elongate.

The genus *Lactobacillus* includes a group of about fifteen recognized species. They are common in fermenting sugar solutions in which they produce lactic acid. Some are of importance in milk and in the ripening of cheese. Some are also of increasing service in biological chemistry, in determination of the presence of certain vitamins and other substances not easily detected by the usual chemical techniques. The species of this genus do not produce the enzyme catalase, in this respect differing from the closely related genus *Microbacterium*[25] which is catalase positive.

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[24] From the Greek *leucus,* colorless, and *Nostoc,* name of a genus of blue-green algae in which the cells occur in chains embedded in a capsular jelly

The genus *Propionibacterium*[26] differs from *Lactobacillus* principally in that it ferments sugar with production of propionic and acetic acids and carbon dioxide rather than lactic acid. Some species are important in cheese ripening.

Species of the genus *Butyribacterium*[27] produce butyric and acetic acids and CO_2 in fermentation of carbohydrates and of lactic acid.

Corynebacteriaceae. This family includes two genera of non-motile and one of motile gram-positive rods, many of them pathogenic.

Corynebacterium[28] has its species characterized as slender, often slightly curved, non-motile rods frequently with a tendency to the formation of club-shaped cells, and with intracellular granules which when stained give a barred appearance to the cell. Many of the species are parasitic, and one, *Corynebacterium diphtheriae*, causes the disease diphtheria in man.

The organisms belonging to the genus *Erysipelothrix*[29] are slender, non-motile and do not grow well in the presence of air; they are micro-aerophilic. They are parasitic in animals and man. *Erysipelothrix rhusiopathiae* is the cause of swine erysipelas.

The species of the genus *Listeria*[30] are small motile rods with peritrichous flagella which produce disease in warm-blooded animals.

Enterobacteriaceae.[31] A large family of gram-negative rod-shaped, non-sporing rods. With it is here combined the family *Achromobacteriaceae*[32] of the Bergey *Manual*, as there seems to be no logical method of differentiation. The family is a very large one containing many genera and numerous species, many of them of great economic significance.

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[25] From the Greek *micrus*, small, and *bacterium*, a small rod or staff. An interesting word which shows how strangely some scientific names are compounded. Both the prefix and the suffix mean small, making a double diminutive.

[26] From the modern Latin word *propionicus*, propionic, in turn from the Greek *pro*, ahead of or first, and *pion*, fat, alluding to the fact that propionic acid is one of the simpler fatty acids; and *bacterium*, a small rod or staff.

[27] From the Greek *butyrum*, butter and *bacterium*, a small rod or staff.

[28] From Greek *coryne*, a club and *bacterium*, a small rod or staff.

[29] From Greek *erysipelas*, red skin or erysipelas, and *thrix*, thread.

[30] Name for Joseph Lister, the British surgeon and bacteriologist.

[31] We are following here the terminology of the Bergey *Manual* (Ed. 6), though it probably is not the name which will eventually prevail. It is inappropriate for at least two reasons. The word *Enterobacteriaceae* means literally "resembling *Enterobacterium*," and there is no contained genus of this name for it to resemble. Further, there are older names some one of which in accordance with the rules of nomenclature should be adopted. The family name *Bacteriaceae* has been used in the past, but as employed in the Bergey *Manual* it includes bacteria which have been inadequately described or which cannot be definitely placed. A committee of British microbiologists has requested an opinion of the International Judicial Commission on Bacteriological Nomenclature which would fix the use of the name *Bacteriaceae* for this family.

The genus *Escherichia*[33] (*Bacterium*) includes certain species which are commonly found in the intestines of man and animals, that are therefore characteristic of domestic sewage, and are in consequence sought in water to determine whether or not it is polluted with sewage. It is differentiated by the active fermentation of the sugar, lactose, with the production of acid and gas, and by its ability to form enough acid under certain standard conditions to produce the acid color in the indicator methyl red. The species most commonly identified is *Escherichia coli* (or *Bacterium coli*), an organism universally studied in the bacteriological laboratory. It is sometimes called the colon bacillus, and organisms of this and closely related genera are termed *coliform bacteria*.

The bacteria of the genus *Aerobacter*[34] are closely related to *Escherichia*, differing primarily in production of less acid under standard conditions so that the color of the methyl red indicator is not changed, the species are said to be methyl red negative. Members of this genus are widely scattered in soil, and surface water and are associated with certain fermentations. A few bacteria closely related to the preceding, found associated with infections of mucous membranes, usually mucoid and with conspicuous capsules, are sometimes placed in the genus *Klebsiella*.[35]

Another genus closely related to the preceding contains species that are usually associated with certain diseases of plants, particularly causing soft rots is *Erwinia*.[36]

The genus *Serratia*[37] is characterized chiefly by the ability of its species to produce pink or red pigments. This property makes the organism quite conspicuous when growing in masses, sufficiently so that the species *Serratia marcescens* which produces the so-called bloody bread was one of the very first of the bacteria to be described and named (in 1823).

[32] Properly spelled *Achromobacteraceae.*

[33] Named for *Escherich*, the bacteriologist. International agreement to use the older generic name *Bacterium* instead of *Escherichia* is probable.

[34] From the Greek *aer*, air or gas, and *bacterium*, a small rod or staff.

[35] Named for the bacteriologist Klebs, with the Latin diminutive ending *-ella*.

[36] Named for the noted plant bacteriologist, Erwin F. Smith.

[37] Named for the Italian, Serrati.

Proteus[38] includes certain species which do not produce pigment and which are actively motile. They are able to ferment glucose but not lactose. Urea is promptly decomposed with the formation of ammonia. A strain of the species *Proteus vulgaris* has been found very useful in the diagnosis of typhus fever.

Salmonella[39] is a large and important genus with species characterized in general by ability to produce acid and gas by fermentation of glucose but not of lactose. Urea is not transformed to ammonia. The genus as now recognized has been put together largely on the basis of certain characteristics to be later discussed under serology. Many of the species and varieties are pathogenic, often associated with food poisoning. One species is the cause of typhoid fever. Because it does not usually produce gas from glucose, it is frequently placed in another genus *Eberthella,*[40] i.e., it is known either as *Eberthella typhosa* or *Salmonella typhosa.*

The genus *Shigella*[41] includes rod-shaped bacteria like *Salmonella,* but non-motile, and producing acid but no gas from glucose. All the species are parasitic, most of them associated with dysenteries, as *Shigella dysenteriae.*

Three other genera somewhat related to those listed above are sometimes placed in a family *Achromobacteraceae.* One of these is the genus *Flavobacterium,*[42] which includes a considerable number of species of motile and non-motile rods, which are non-parasitic, do not ferment carbohydrates actively, and produce definite yellow to orange pigments. They are common in water and in soil. *Achromobacter*[43] was created as a genus to include similar bacteria which produced little or no pigment. They likewise are common in water. The third genus *Alcaligenes*[44] contains organisms which produce no acid from carbohydrates but are characterized by production of an alkaline reaction when grown in milk. The species are found in the intestinal tract of vertebrate animals, and some in dairy products. For example, *Alcaligenes viscosus* is a common cause of slimy or ropy milk.

[38] From the Greek sea god *Proteus* who often changed his form.

[39] Named for Salmon, the American bacteriologist.

[40] Named for Eberth, a German bacteriologist, with Latin diminutive ending *-ella.*

[41] Named for the Japanese bacteriologist Shiga, with Latin diminutive ending *-ella.*

[42] From Latin *flavus,* yellow, and Greek *bacterium,* a small rod or staff.

[43] From the Greek *achromus,* colorless, and *bacterium,* a small rod or staff.

[44] From the modern Latin *alcali,* alkali, in turn from the Arabic *al qualey,* the ashes of certain desert plants, and the Latin *gigno,* to produce.

Parvobacteriaceae.[45] This family includes organisms which are gram-negative rods, usually parasitic in warm-blooded animals, often pathogenic. Many require the presence of body fluids or specific growth-promoting substances when grown outside the animal body. Several of the genera include organisms which when grown in suitable environment and stained show heavier staining at the ends of the cell, they are characteristically bipolar.

Four groups of genera have been recognized. The first of these (tribe *Pasteurelleae*) includes three genera, all of which show some tendency to bipolar staining. The genus *Pasteurella*[46] includes certain highly pathogenic organisms, such as that of bubonic plague (*Pasteurella pestis*) in man. *Malleomyces*[47] usually shows short rods with bipolar staining, and a tendency to form filaments and even branched cells. The species are pathogenic, including *Malleomyces mallei* of glanders in the horse. The species of the genus *Actinobacillus*[48] have cells which are typically rod-shaped but which show much pleomorphism from spheres to short filaments. All are pathogenic.

The second tribe *Brucelleae* includes the single genus *Brucella*[49] in which the cells are short without decided tendency to bipolar staining, usually pathogenic, including such organisms as those causing undulant fever (*Brucella melitensis*) and of abortion in cattle (*B. abortus*).

The tribe *Bacteroideae* includes bacteria closely related to the preceding, but not growing when in contact with air, i.e., they are obligate anaerobes. There are two genera. *Bacteroides*[50] includes a considerable number of species of bacteria with rod-shaped cells in which the ends of the cells are not pointed, but rounded. The species inhabit the bodies, particularly the intestines, of man and animals, and some are apparently pathogenic. The genus *Fusobacterium*[51] includes rod-shaped anaerobic bacteria in which the cells are pointed or somewhat tapering. All are parasitic, usually in the mouth.

[45] The name is inappropriate, as there is no contained genus *Parvobacterium* Many of the organisms contained are by no means small as compared with other bacteria. From the Latin *parvus*, small, and *bacterium*, small rod or staff. It is a double diminutive, and has the further disadvantage of being a Latin-Greek hybrid word.

[46] Named for Pasteur, the eminent French bacteriologist, with Latin diminutive ending *-ella*.

[47] From the Latin *malleus*, the disease glanders of the horse and the Greek *myces*, a fungus.

[48] From the Greek *actis*, *actinis*, ray, and Latin *bacillus*, a small rod

[49] Named for *Bruce*, the British bacteriologist, with Latin diminutive ending *-ella*

[50] From the Greek *bactrum*, rod or staff, and *eidus*, resemblance

[51] From Latin *fusus*, a spindle, and the Greek *bacterium*, a small rod or staff

The tribe *Hemophileae* includes four genera. All are parasitic and when first grown in the laboratory upon isolation require the presence of growth stimulants not present in the usual peptone broth, such as those present in hemoglobin, ascitic fluid, or other body fluids or in certain plant juices. The species of *Hemophilus*[52] are aerobic or facultative, are non-motile, and occur singly, at least not predominantly in pairs. *Hemophilus influenzae* is commonly found associated with certain respiratory infections in man. In the genus *Moraxella*[53] the non-motile cells occur frequently in pairs or chains. One species *Moraxella lacunata* is associated with a type of conjunctivitis in man. The cells of organisms in the genus *Noguchia*[54] are motile, in some species with a polar flagellum, in another peritrichous. The *Noguchia granulosis* is the probable cause of the human eye disease, trachoma. The genus *Dialister* includes two species of organisms which require exclusion of oxygen when grown in cultures. They are found in mucous membranes.

Rhizobiaceae. The most characteristic genus of this family is *Rhizobium,*[55] which includes the bacteria which produce nodules in the roots of leguminous plants, and during growth therein and in combination with the higher plants fix the nitrogen of the air, that is, combine it into compounds. These bacteria are gram-negative, without spores, and usually with one to few flagella. They grow well in the presence of air (oxygen). Two other genera are included in this family in Bergey's *Manual,* 6th ed. Their relationships to *Rhizobium* are questionable. These genera are *Agrobacterium,*[56] to include certain species of bacteria causing galls on plants or other hypertrophies on stems or roots, and *Chromobacterium,*[57] which may be readily differentiated in that each of the several species produces a violet pigment when grown under suitable conditions.

Nitrobacteraceae. The bacteria of this family are for the most part soil and water forms which are able to utilize carbon dioxide from the air and produce the organic compounds needed for their cell growth. They are said to be autotrophic. As a part of their physiological processes they apparently secure growth energy by the oxidation of certain elements or inorganic compounds, they may produce thereby changes significant in soil fertility. Three groups of genera may be recognized, most of them inadequately studied and understood. All the organisms belonging to the first group oxidize ammonia to nitrites or nitrites to nitrates. Among the genera are *Nitrosococcus*[58] and *Nitrobacter*.[59] The organisms of the genus *Hydrogenomonas*[60] are able to oxidize elementary hydrogen, and those of the genus *Thiobacillus* oxidize elementary sulfur and the thiosulfates.

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[52] From the Greek *haema,* blood, and *philos,* loving.

[53] Named for the microbiologist Morax with Latin diminutive ending *-ella.*

[54] Named for the Japanese bacteriologist Noguchi.

[55] From Greek *rhizus,* root, and *bius,* dweller.

[56] From Latin *ager,* field, and Greek *bacterium,* small rod or staff.

[57] From Greek *chromus,* color, and *bacterium,* small rod or staff.

2. THE ORDER ACTINOMYCETALES

The Ray Bacteria (or Ray Fungi)

The organisms of the order *Actinomycetales* include those bacteria which have certain resemblances to the fungi and are often considered to be intermediate between the molds and the true bacteria. The cells are elongated and relatively slender. In some genera the cells become greatly elongated and branch, producing a mat of branched threads resembling the mycelium of a fungus. The three families are the *Mycobacteriaceae*, the *Actinomycetaceae*, and the *Streptomycetaceae*. A key for the differentiation is given in the Appendix.

Mycobacteriaceae. This family includes a single genus, *Mycobacterium*.[61] The cells are usually slender rods which stain with dyes with some difficulty, but when once stained they resist removal of the stain by the application of acid, in this differing from other bacteria. These bacteria are said to be *acid-fast*. The cells are sometimes swollen, club-shaped, and even branched. Included in this genus are some pathogenic species such as *Mycobacterium tuberculosis*, the cause of tuberculosis in man and animals, and *M. leprae*, the probable cause of leprosy.

Actinomycetaceae. The organisms of this family produce elongate much-branched cells forming a mycelium much like that of molds, but with the threads much more slender. After a time these threads break up into segments, rod-shaped or spherical, these cells function as spores, developing into mycelium when brought under favorable environment. In some cases buds develop laterally on the mycelial threads, and also function as spores. There are two genera. *Actinomyces*[62] includes those species which grow in the absence of oxygen

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[58] From modern Latin *nitrosum*, nitrous, and Greek *coccus*, grain or berry.

[59] From Latin *nitrum*, niter, and Greek *bactrum*, rod or staff.

[60] From modern Latin *hydrogenum*, hydrogen, and Greek *monas*, *monadis*, a unit, a monad.

[61] From the Greek *myces*, a fungus, and *bacterium*, a small rod or staff

[62] From the Greek *actis*, *actinis*, a ray, and *myces*, a fungus

(anaerobic or microaerophilic) The best known species is the *Actinomyces bovis,* the cause of lumpy jaw in cattle. The genus *Nocardia*[63] is made up of numerous species which grow well in the presence of air. Some of the species show some slight tendency to be acid-fast, resembling in this respect the genus *Mycobacterium.* Some, such as *Nocardia farcinica* which produces the disease bovine farcy, are pathogenic; many occur in the soil.

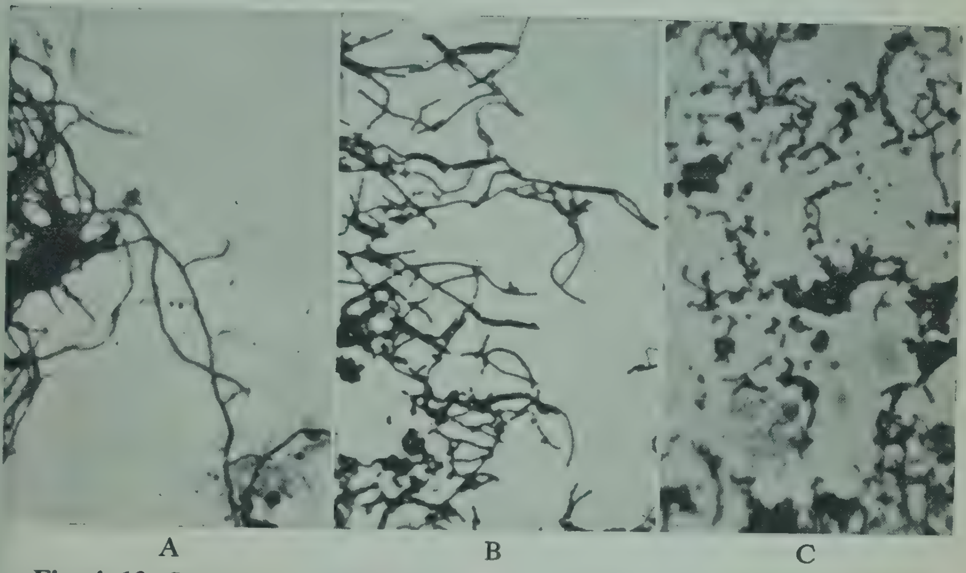

Fig. 4–13. *Streptomyces.* Photomicrographs. A. Branching mycelium. B. The segmentation of the mycelium and the aerial hyphae into spores. C. Fragmentation into spores practically completed. Note that the chains of spores in B and C are straight.

Streptomycetaceae. The organisms belonging to this family likewise produce a much-branched mass of threads or mycelium, usually of two types. That mycelium which grows in and on the food material (the vegetative mycelium) is much-branched, but does not tend to break up into segments readily as in the family *Actinomycetaceae.* In the genus *Streptomyces*[64] a part of the threads grow into the air, forming an aerial mycelium, where they develop spores of the type generally termed conidia. These aerial spores are usually produced in chains. Most of the species described have been isolated from soil. Several have been found to be of significance because of their ability to produce antibiotics, such as *streptomycin* from *Streptomyces*

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[63] Named for the bacteriologist Nocard.

[64] From Greek *streptus,* a thread, and *myces,* fungus.

griseus. The second genus, *Micromonospora*,[65] does not produce the luxuriant aerial mycelium of *Streptomyces* but forms instead short spore-bearing threads on the surface, each bearing a single terminal spore or conidium. The organisms are found in soil, lake mud, and in heating composts.

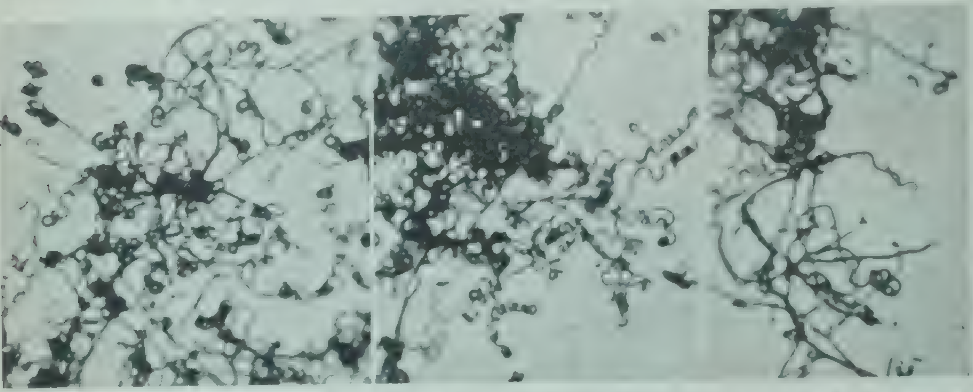

Fig. 4–14. *Streptomyces*. Photomicrographs. Three illustrations of the aerial spirals formed by some species, showing also the segmentation of the spiral threads into spores.

3. THE ORDER SPIROCHAETALES

The Spirochetes

The bacteria belonging to this order show certain characteristics which have caused some students to place them with the protozoa. They differ from most other bacteria in that the cells are flexuous, and motility results from the bending motions of the cell. In most cases the cell is definitely curved, showing at least one turn of a spiral. The several species show great variations in length, from a few microns to several hundred microns. No flagella have been reported for most species; the electron microscope seems to indicate their presence in some cases. Most of the species do not stain readily with the aniline dyes usually used with bacteria; they require special techniques for observation. Two families are recognized, the *Spirochaetaceae* and the *Treponemataceae*.

Spirochaetaceae. The cells are relatively coarse. The genera *Spirochaeta*[66] and *Saprospira*[67] include free-living water species. The genus *Cristispira*[68] is made up of species which occur in a special organ

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[65] From the Greek *micrus*, small, *monos*, single, and *spora*, seed, or in modern Latin, a spore.

[66] From the Greek *spira*, that which is coiled, and *chaete*, long hair.

[67] From Greek *saprus*, rotten or decayed, and *spira*, a coil or spiral

[68] From the Latin *crista*, a crest, and Greek *spira*, a coil or spiral

(the crystalline style) of certain molluscs. None is of economic importance.

Treponemataceae. The organisms of this family are slender (sometimes coarse) spirals from 4 to 16 μ in length. These organisms are parasitic on animals, many are disease-producing. The genus *Borrelia*[69] has cells which are somewhat coarse, with few shallow spirals tending to taper at the ends into fine filaments. They are unique also in staining readily with the usual aniline dyes. Some are the causes of blood infections and relapsing fevers in man (*Borrelia recurrentis*), animals, and birds. The other two genera contain species more difficult to demonstrate by use of stains. The cells are also relatively more slender. The genus *Treponema*[70] includes the species which grow only with exclusion of oxygen. All are parasitic and most are disease-producing, such as *Treponema pallidum,* the cause of syphilis. The genus *Leptospira*[71] includes also slender spiral organisms, which however require oxygen for growth in culture. The cells are finely coiled, with one or both ends bent into a semicircular hook. Infectious jaundice in man is caused by *Leptospira icterohaemorrhagiae*.

4. THE ORDER MYXOBACTERALES

The Slime Bacteria

The bacteria of this group have been studied comparatively little. They differ from the other bacteria in having a surprisingly complex life cycle. The individual cells resemble those of the true bacteria and are relatively slender. They are somewhat flexuous and are able to move (crawl) about on the surface of the substrate, though they do not possess flagella. They excrete a large amount of gelatinous material forming slimy colonies or masses. When conditions are right, this *mass develops* into a relatively complex fruiting body. In some cases a cyst or groups of cysts is formed with the bacteria in the interior. In some species these cysts form on the ends of stalks, the whole structure having a superficial resemblance to the slime molds, a group sometimes placed with the fungi and sometimes with the protozoa. The cysts when dry may be blown about and appear to function as spores. When a cyst is placed under conditions favorable for growth, water is absorbed, the cyst swells and bursts, and the bacterial cells swarm (crawl) out and establish a new colony. In some cases the rod-shaped cells shorten and become spherical during the process of encystment and are termed spores. On germination they again become rod-shaped. The group is sometimes termed the *Polyangidae* after the first genus (*Polyangium*) described.

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[69] Named for the microbiologist Borrel.

[70] From the Greek *trepo,* to turn, and *nema,* thread.

[71] From Greek *leptus,* thin or fine, and *spira,* a coil or spiral.

The order *Myxobacterales* is relatively large, including five families, thirteen genera, and many species. The bacteria of this order are widely distributed in soils and are particularly common on manure. These organisms have not been sufficiently studied to determine whether they have any special significance in the soil. Several species have been described as parasitic on fish, others are of significance in the decomposition of cellulose. Some parasitize other species of bacteria.

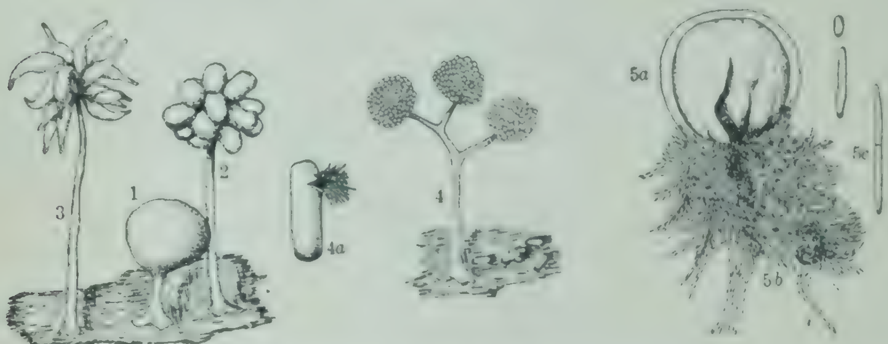

Fig. 4–15. Some typical members of the order *Myxobacterales*. 1, 2, 3. Fruiting bodies; the bacterial colony has produced a stalk surmounted each by from one to several cysts, each containing spores. 4. A species in which the cystophore or stalk branches. *4a*. A cyst germinating, showing the escape of the young colony. *5a*. Germinating cyst from which the bacterial cells (*5b*) are crawling. *5c*. Isolated cells of the colony, showing multiplication by fission.

5. THE ORDER CHLAMYDOBACTERIALES

(The Sheathed Bacteria)

The bacteria which belong to this order are frequently designated as the iron bacteria. For the most part they are filamentous forms in which the sheath becomes heavily coated with iron in the form of the hydroxide or carbonate. The one family, the *Chlamydobacteriaceae*, includes several genera. None of them is commonly grown in the laboratory. Some species belonging to the genera *Leptothrix* and *Cladothrix* are common in water containing considerable amounts of iron.

6. THE ORDER RICKETTSIALES

The Rickettsias or Rickettsiae

The organisms belonging to this order have become adapted to a high degree of parasitism, they are found in or on living cells of animals and of various arthropods, quite probably of other forms of animal life as well. The organisms are usually grown in the laboratory in living tissues or embryonated eggs. Many are disease-producing. The cells are minute, rod-shaped, spherical, or irregular. In mode of life they most closely resemble the filterable viruses, in some respects show resemblances to certain of the parasitic protozoa. The order is important in that it contains many species which produce disease or are parasitic in man or animals. In many genera the organisms are transmitted to the vertebrate host by lice, fleas, mites, ticks, and perhaps by other arthropods. Three families have been recognized.

The family *Rickettsiaceae* includes organisms which seem to be characteristic of arthropods, growing in the gut and in its walls. Some species are transmitted by the bite of lice, ticks, etc., to man and animals, producing serious diseases. Here they grow as intracellular parasites.

At least three genera are recognized. In *Rickettsia*[72] the cells are small, often variable in shape, rod-shaped to spherical, occurring in the cytoplasm of lice, fleas, mites, and ticks. *Rickettsia prowazekii,* the cause of typhus (not typhoid) is transmitted usually by the head or body louse. The genus *Coxiella,*[73] with one species, *C. burnetii,* differs primarily from *Rickettsia* in being sufficiently small in some growth stages to pass through the pores of a porcelain filter. It is the cause of Queensland (Q) fever in man. The genus *Cowdria*[74] (one species *C. ruminantium*) closely resembles *Rickettsia,* is transmitted by the bite of a tick, and is the cause of the disease heartwater in sheep, goats, and cattle.

The family *Chlamydozoaceae* includes small, usually coccoid or pleomorphic microorganisms. They occur only as parasites in the cytoplasm of cells. Organisms which are coccoid and cannot be cultivated in chicken embryos are placed in the genus *Chlamydozoon.* An example is *Chlamydozoon*[75] *trachomatis,* the cause of trachoma in man. The genus *Miyagawanella*[76] closely resembles *Chlamydozoon* but may be cultivated in chick embryos. Several diseases of man and animals are caused by species of this genus, such as psittacosis, a disease in birds, transmissible to man. The genus *Colesiota*[77] includes one species, *C. conjunctivae,* which causes disease of the conjunctiva of the eye in sheep, cattle, and goats.

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[72] Named for the American bacteriologist Ricketts.

[73] Named for the microbiologist Cox with Latin diminutive *-ella.*

[74] Named for the microbiologist Cowdry.

[75] From the Greek *chlamys, chlamydis,* a cloak, and *zōum,* living being or animal.

The family *Bartonellaceae* includes species which generally attack the red blood cells in man and other mammals. The cells may be of various shapes. The genera are *Bartonella, Haemobartonella, Grahamella,* and *Eperythrozoon. Bartonella*[78] has been cultivated in serum media. Cells are motile. The single species *Bartonella bacilliformis* is the cause of Oroya fever.

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[76] Named for the Japanese bacteriologist Miyagawa with the Latin diminutive ending *-ella.*

[77] For the microbiologist Coles with the Greek suffix *iota*—iota, the smallest letter in the Greek alphabet.

[78] Named for the microbiologist Barton with the Latin diminutive ending *-ella.*

CHAPTER 5

The Viruses, Their Characteristics and Classification

The term *virus* as used currently in bacteriology is applied to a group of particles (or organisms) usually though not invariably ultramicroscopic, which increase only in the presence of cells of plants (including the bacteria) or animals. They make their presence known by the production of various abnormalities in the hosts; so far as is known they all cause disease, and injure or destroy the cells which they invade.

The word *virus* was employed by the Latins with several meanings, among them that of a poison or venom. With the development of the germ theory of disease the term virus came to be used as a general designation for the cause of any infectious disease. Then it was discovered that the infective agents could be divided into two groups, those sufficiently large to be observed and studied by use of the microscope and those in which microscopic study failed to reveal microorganisms. Studies then showed that organisms as large as the bacteria could be removed from liquids by filtration, usually through unglazed porcelain, but that particles capable of producing certain diseases were small enough to pass through such filters. Indeed the French bacteriologist Pasteur suggested that there might be ultramicroscopic objects (or organisms) capable of producing disease. In his study of rabies, he was able to transfer the disease from animal to animal indefinitely by injection of infective material taken from a diseased animal, but he was quite unable to find any organisms by microscopic observation.

Satisfactory proof that there do exist ultramicroscopic agents capable of disease production was brought forward by Iwanowsky who worked with plants affected with the disease known as tobacco mosaic. He crushed and pressed the tissues of the diseased plant and passed the expressed juice through a filter with pores sufficiently fine to remove microscopically visible bacteria. Injection of this filtrate into a suitable plant regularly reproduced the disease. The unknown agent in the filtrate capable of inducing disease was evidently a *filtrable*[1] *virus*.

Later Loeffler and Froesch (1892) proved that foot and mouth disease of cattle and sheep is caused by a filtrable virus and concluded that it was ultramicroscopic. These discoveries opened the way to development of proof that many important diseases of plants and animals are caused by filtrable viruses.

Later Twort and d'Herelle independently found that somewhat similar agents were able to destroy, even to dissolve, bacteria, and that these were also filtrable and ultramicroscopic. To them the name *bacteriophage*[2] was given.

That the living cells of plants and animals of many groups may be attacked by filtrable viruses[3] with production of injury or death has been well established. So many and so important are the diseases that their study has led to the development of *virology* as a distinct subdivision of bacteriology.

Whether there are agencies of the size and nature of viruses which are able in nature to bring about changes in non-living substances has not thus far been satisfactorily demonstrated. The only viruses now known are those associated with living cells and detectable primarily by the injury which they produce. Thus far we have no very definite evidence that ultramicroscopic organisms bring about extracellular fermentative and other changes corresponding to those produced by bacteria, fungi, and protozoa.

Characteristics of Viruses

Nature of the Viruses. The development and use of the electron microscope has done much to clear up the mystery relative to the morphology of the viruses. We now are able to observe by means of the electron photographs and the fluorescent screen the size and shape of the virus particles.

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[1] Or *filterable virus*. Perhaps *filtrable* is preferable etymologically, but both spellings are used in the literature.

[2] From the Greek *bacterium*, a small rod, here used in the technical sense of the bacteria as a group, and *phagein*, to devour.

[3] Occasionally the term *virus* is used to designate the causal agent of an infectious disease before its nature has been determined. However, in recent bacteriological literature *virus* has come to be used for *filtrable virus* and this meaning will be adhered to in this volume.

Are viruses living things or do they resemble complex organic compounds of the general nature of the enzymes generally regarded as non-living? Obviously the answer will be determined in part by the definition we may formulate as to the meaning of "living," and upon the adequacy of our knowledge of the virus. The following facts seem to be well established.

Viruses Are Particulate. Some of the earlier investigators thought the viruses might not be made up of definite particles—perhaps they were substance in solution which caused not only injury of the cell attacked but likewise stimulated the production by the cell of more virus of the same kind. Soon, however, there developed evidence from many sources that a virus is made up of particles. Centrifugation of suspensions with determination of the rate of sedimentation of the virus particles makes it possible to estimate their size. This may also be determined by use of filters with different pore diameters. It is possible to count the numbers of virus particles (particularly bacteriophage) by methods analogous to those used in the estimation of the number of bacteria in milk and water and by the infectivity of dilutions. So even before the advent of the electron microscope it was established that the viruses are particulate, and that a definite size of particle was a characteristic of each virus.

The electron microscope has facilitated the more accurate determination of the size and shape of the virus particles. In general the particles of a single virus were found to be relatively uniform as to size and shape. Some were long rods, others short rods, some were spherical and some, as many bacteriophages, were spherical, each with a relatively long slender appendage.

Size of Virus Particles. All viruses are very minute although some are large enough so that they may be recognized by special illumination, as with the phase microscope, at the highest magnifications attainable with visible light. This means that they are in general smaller than one-tenth of a micron in diameter, that is, less than one ten-millionth of a centimeter. Measurements in this range of sizes are usually made by use of the unit of measurement, the millimicron (abbreviated $m\mu$; $1000\ m\mu = 1\ \mu$).

The rod-shaped or elongate viruses thus far studied vary in diameter from $10\ m\mu$ to $20\ m\mu$, and from 20 to $400\ m\mu$ in length; the spherical forms from $8\ m\mu$ to $250\ m\mu$. Their minuteness is emphasized when we find that in general they are far smaller than a bacterial cell.

In fact, a bacterial cell of a cubic micron would have a volume equal to the combined volume of 1 million virus particles each with a diameter of 10 mμ.

Morphology of the Virus Particles. As noted above, the virus particles may be rod-shaped or spherical. There is some evidence that the particles are not always homogeneous in structure. Many of the bacteriophages are characterized by the possession of a slender, straight, or somewhat curved appendage, giving the appearance of a head and tail. The "tail" has a diameter only a fraction of that of the head. The "tail" is apparently not an organ of locomotion. There is no evidence that the virus particles are motile. With a few viruses there has been found some evidence of the presence of an outer membrane, and perhaps some internal differentiation.

Virus Crystals. Stanley developed chemical techniques for flocculation or precipitation of virus particles from a suspension. Resuspension and reflocculation made possible the securing of viruses of certain kinds, as the virus of tobacco mosaic, in relatively pure condition. When the purification occurred under suitable conditions, the virus appeared in the form of a crystal, resembling the crystals of protein which have long been known. This fact that virus could be crystallized was regarded by some students as good evidence that the virus particles could not be living. However, the facts that the virus particles retained their identity and that the crystal represented merely an orderly arrangement of virus particles have made it evident that the phenomenon of crystallization is by no means conclusive evidence of the inanimate character of the particles.

Composition of Virus Particles. Chemical studies have shown that virus particles are composed principally of complex protein. The amino and nucleic acids identified by the digestion of the virus particle hydrolysates are the same as those which constitute building blocks in the proteins of plants and animals generally.

It has been suggested that a virus particle might be regarded as a single giant protein molecule. However, small as a virus particle is, it nevertheless is large when compared with most protein molecules. Chemical analyses have shown that at least a considerable portion of the virus particle is made up of nucleoprotein. The studies of Luria and his co-workers with the virus called bacteriophage T_2 seem to indicate that there may be within a single phage particle as many as 25 units that have the characteristics of genes. Evidently the phage particle may have a somewhat complex organization and may not therefore consist (as has sometimes been postulated) of a single pure protein. The mass of the T_2 phage has been calculated to be 2.5×10^{-16} gram. Of the phage about 4.5 per cent is phosphorus.

Growth and Reproduction of Virus Particles. Thus far no virus has been reported as having been grown entirely independently of living cells of plants or animals. Viruses are obligate parasites, in this character resembling certain of the bacteria such as members of the genera *Rickettsia, Bartonella,* and *Coxiella.* When in the right cellular environment the virus particles may increase in numbers with extraordinary rapidity.

The minuteness of the virus particles and the fact that they cannot be observed under the electron microscope except when dried and in a vacuum has thus far prevented direct observation of their method of increase. This has made difficult the problem of giving any authoritative answer to the question "Are the viruses living organisms?" Perhaps the best criterion of whether a thing is living is its ability to take up and utilize food. A living organism in contact with suitable nutrients should be able to transform the nutrients through the activity of its numerous enzymes and build up the needed constituents of the living cell. A living cell does not grow like a crystal simply by accretion of similar particles from its environment. It remodels the nutrients of the environment to produce its own cell constituents. By this production and incorporation of new body substance the cell grows in size. Increase in number of cells is by the formation by budding or splitting or segmentation of new organisms. Is this the way that viruses multiply? There does not seem to be enough evidence as yet to constitute a positive proof. If they do grow and multiply in this fashion they may well be called living organisms.

Some investigators have postulated another method of increase of virus particles. They believe that the virus may be regarded as a huge enzyme molecule which in the living cells of its hosts catalyzes the production of other molecules of the same type as the virus. In this case the virus particles would not assimilate and metabolize food materials, multiplication would not be by increase of a particle with its ultimate division.

Research in this field is active, and an adequate answer to the question "Are viruses alive?" may be expected.

From a practical point of view it seems wise tentatively to regard the viruses as ultramicroscopic living organisms, differing from larger organisms in details, but fundamentally resembling them. This has been well stated by Burnet (1945):

> For some purposes it may be desirable to allocate them to a new category sharply differentiated from all other living organisms, but as far as the doctor, the public health administrator, and the biological experimenter are concerned, the pragmatic necessity will remain that viruses be regarded as organisms—self reproducing, varying, and surviving like any other living being.

Several theories as to the origin and relationships of the viruses have been developed. Emphasis has been laid upon the fact that they have never been grown or cultivated independently of living cells. It is well known that normal living cells contain particles which have certain of the characteristics of viruses, but are not only non-injurious to the cell but are apparently essential. The *genes* of the chromosomes of a cell nucleus, so significant in inheritance, are believed to be of about the size of a virus particle. There are also to be found in the cytoplasm of cells the mitochondria and microsomes which are about the same size or larger. There is evidence of the occurrence in the cytoplasm of cells of so-called plasmogenes which are apparently significant in inheritance of certain types of characters. Most significant is the fact that these particles in the cell grow and multiply. Certainly they are generally regarded as living. They may be thought of as constituting an order of living things more elementary than the cell. One may conceive of living things as of several orders of complexity. A bean plant is a complex organism made up of many cells, of many kinds, all together constituting the individual. However, among the unicellular animals or plants, the protozoa, or bacteria, a single cell constitutes the individual, each however containing many distinctive living units or organellae. Perhaps the viruses may be considered to be living objects at the level of these cell organellae. Just as some kinds of bacteria and protozoa live only in or on other cells, so the virus particles may constitute a parasitic group of a more primitive, or at least simpler, order than the cell.

Another concept of viruses is that they have originated from other organisms by a process of degeneration. This has been summarized by Burnet:

> Viruses are micro-organisms which have evolved by parasitic degeneration from larger micro-organisms, many of them in all probability from

bacteria. They show evidence in their chemical structure of conformity with the general pattern of living material, and their behavior must be interpreted mainly in terms of biological concepts.

Cultivation of Viruses. Again it may be emphasized that thus far it has not been found possible to increase or cultivate viruses except in cells or living tissues of suitable animals or plants.

Some plant viruses are highly selective, producing disease, i.e., growing, in only a single kind of plant; other plant viruses may be capable of growing in many, even in hundreds of kinds of plants, and in those belonging to distantly related families. Cultivation of the virus is possible only by introducing it into the right plant under the right conditions. This is not always a simple procedure as will be noted later in consideration of the virus diseases of plants.

Similarly an animal virus may adapt itself to many species of animals, as is true of rabies virus, or it may attack only a single species. Further, animal viruses may grow only in certain tissues of an animal, some grow best in nerve tissues, others in cells of certain membranes or glands, others will grow almost anywhere in the animal body. In many cases cultivation of viruses has been much simplified by the fact that the growing tissues of the embryo chick are suitable for their growth.

The bacteriophages are frequently highly specific, attacking and growing in a single kind of bacterium, or even limited to a single strain. The comparative ease of growing bacteriophage has led to a great amount of study on this group of viruses.

The statement that viruses will grow and multiply only in living cells may require some modification. It has been found possible so to treat bacteria that they cease multiplication but remain susceptible to attack by bacteriophage and permit multiplication of the phage particles.

Relationships of Viruses to Environment. Viruses may be destroyed by high temperatures and by many of the same chemical compounds that destroy bacteria. They may also be killed (destroyed) by irradiation just as are larger microorganisms.

The temperatures and times required to destroy a virus may be quite different from those required to kill the host cell. For example, the virus which causes the disease aster yellows in many plants is transmitted from plant to plant by certain insects (leaf hoppers). The virus in leaf hoppers may be destroyed by subjecting them to temperatures which do not kill the insects. The virus that causes peach yellows may also be destroyed by exposure to low temperatures but somewhat higher than that required to kill peach buds. Kunkel (1947) concludes "The thermal reactions of these viruses do not suggest that they have been derived from normal plant proteins, from genes, or from plasmagenes. The heat treatment experiments confirm the view that plant viruses are autonomous and unrelated genetically to their host plants."

Viruses resemble other microorganisms in showing a marked degree of stability combined with some degree of variability. For example, the virus of one type of tobacco mosaic will reproduce the same disease through many generations of inoculations. However, by suitable irradiation, it has been possible to secure strains of the virus, apparently mutants, which produce a disease with different symptoms. In this respect again the viruses resemble the bacteria. Furthermore, a virus may in its several strains exhibit marked difference in pathogenicity, or disease-producing power. Mutations in bacteriophage have frequently been noted.

CLASSIFICATION AND NAMING OF THE VIRUSES

The first names given to viruses were based generally upon the disease produced or upon the name of the animal or plant susceptible. Among these were virus of foot and mouth disease of cattle, virus of mumps, virus of hog cholera, virus of aster yellows, virus of cucumber mosaic, and virus of poliomyelitis. As noted above, those attacking bacteria were generally grouped under the name *bacteriophage*, or more briefly, *phage*.

There are at present two schools of thought with reference to the naming of the viruses. One believes that for the present, at least, and until there is more known relative to the characteristics of the viruses a simple nomenclature based upon hosts and disease characters should be maintained. The other school contends that the viruses so closely resemble other forms of life that there is no reason for proposing any form of nomenclature other than that accepted for all living things, the binomial or Linnaean system.

Place of the Viruses in the Classification of Microorganisms. Two views have been suggested. One is that the viruses are closely enough related to the bacteria, i.e., to the Class *Schizomycetes* that they should be included in this class as an order named the *Virales*. This is the view taken by Holmes in the sixth edition of the Bergey *Manual*.

The second concept is that for the present at least it is better to keep the viruses separate from the *Schizomycetes*, at least recognize them as a distinct class. Possibly they are sufficiently distinct even to be placed in a separate kingdom on the basis that they are essentially subcellular rather than cellular in organization. This disposition was suggested in an earlier publication by Holmes, who named the group the *Vira*.[4] In this text the *Vira* will be regarded as a class coordinate with the *Schizomycetes*.

The Class Vira

The Class *Vira* includes a single order, the *Virales*. In general, the outline of the classification as outlined by Holmes in the sixth edition of the Bergey *Manual* will be followed.

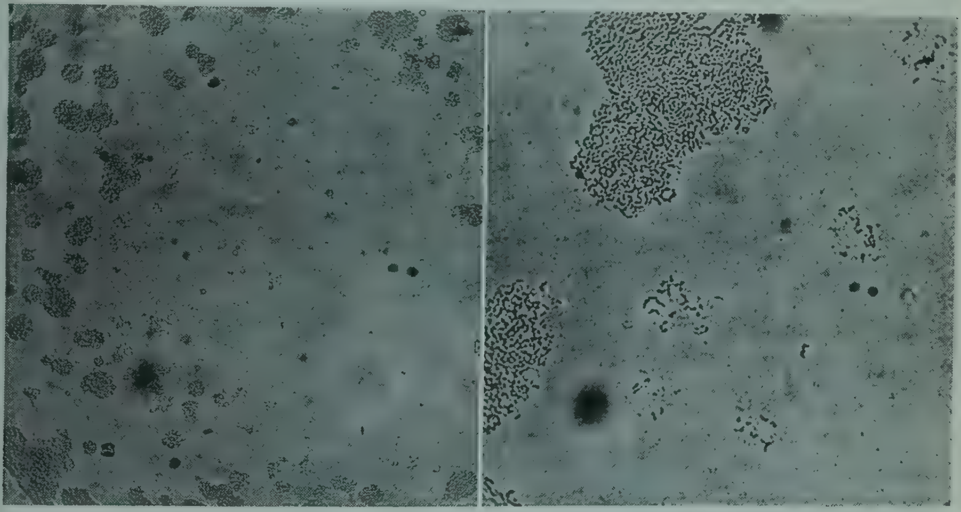

Fig. 5–1. Plaque showing bacteriophage action on plates with colonies of *Streptococcus lactis*. A. Normal colonies surrounding an area (plaque) in which the colonies are undergoing lysis. B. Upper colony nearly normal, showing chains of cocci. Below several colonies in process of lysis, a few organisms still visible, the remainder dissolved. (From Parmelee, Nelson, Turner, and Carr. Courtesy *National Butter and Cheese Journal.*)

It is quite possible that with studies of the virus particles that morphology will be increasingly significant in classification. However, thus far classification is largely based upon the kinds of hosts, the diseases and lesions produced in the animals or plants infected, the methods of disease transmission, particularly the transmitting agents such as insects, mites, and ticks, and the ability of the virus to withstand unfavorable conditions.

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[4] Names of higher groups such as classes are words in the plural. Holmes wished to use the plural of *Virus* for this name. The Latin word *virus* is a neuter noun which so far as the records show had never been used in the plural. The ending *-us* is very uncommon on a neuter Latin noun; there are only a half dozen such. On the basis of competent etymological authority he proposed that the plural Latin form *vira* be recognized and adopted. He also proposed the name *Vira* for the scientific designation of the entire group of viruses, and as constituting a kingdom coordinate with the Plant and Animal Kingdoms.

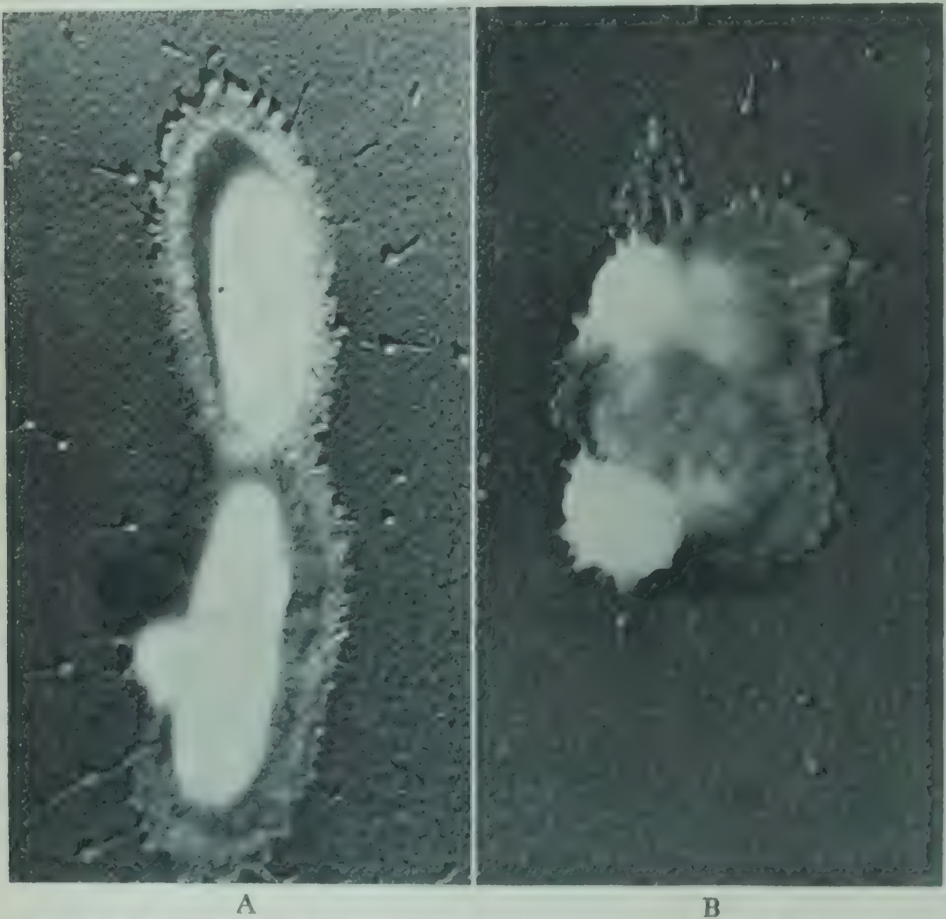

Fig. 5–2. Bacteriophage and its action on *Streptococcus lactis*. A. Two elongate cells of *S. lactis* attacked by phage. The protuberance on the lower cell may be an indication of incipient lysis. B. Two cells of *S. lactis* in which lysis is more advanced. (Courtesy of Parmelee, Carr, and Nelson and of *Journal of Bacteriology*.)

The viruses may be separated into three natural groups,[5] regarded as suborders of the *Virales;* those which attack the bacteria, the

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[5] *Phagineae* is from the Greek *phagein*, to devour, the same root as is found in *phage* and *bacteriophage*. *Phytophagineae* from the Greek *phytum*, plant, means literally the plant phages. *Zoophagineae* from the Greek *zoon*, animal, means animal phages.

Phagineae or bacteriophages; those which attack the higher plants, the *Phytophagineae;* and those which grow in the cells of animals, the *Zoophagineae.*

The *Phagineae* have a single family, the *Phagaceae,* and this in turn a single genus, *Phagus.* Some of the best-known types are revealed by the electron microscope to be spherical with an elongate appendage like a tail. The *Manual* describes 46 species, the type species one of those parasitizing *Escherichia coli* is named *Phagus minimus.* It is probable that if host specificity is accepted as a basis for the differentiation of bacteriophage species there may readily be thousands of species.

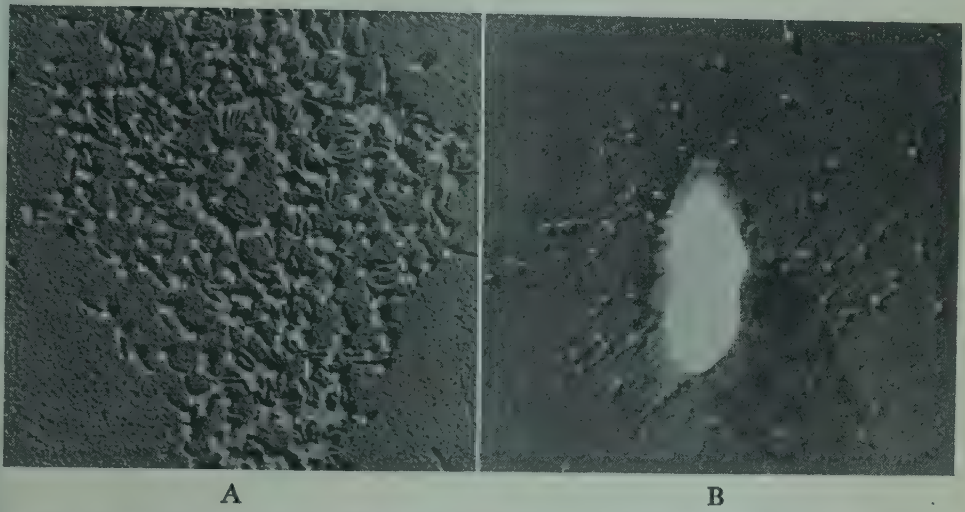

Fig. 5–3. Bacteriophage. Electronographs. A. Bacteriophage. Note that the phage particles have been "shadowed" to bring out their morphology. Each is made up of a nearly spherical (occasionally elongate) "head" and a filamentous straight or curved appendage or "tail." The head has a diameter of about 70 mμ, with a tail approximately 30 mμ in diameter and 150 mμ in length. B. A normal dividing cell of *Streptococcus lactis,* showing constriction and some marginal evidence of capsular material, surrounded by particles of phage. (After Parmelee, Carr, and Nelson. Courtesy *Journal of Bacteriology.*)

The *Phytophagineae* include a large number of viruses of higher plants, some of them of great economic importance. Holmes, in the Bergey *Manual,* includes the six families, *Chlorogenaceae* or yellows disease viruses, with six genera and twenty-eight species, *Marmoraceae* or mosaic viruses, with six genera and eighty-five species, *Annulaceae* or ringspot viruses, with one genus and seven species, *Rugaceae* or leaf curl viruses, with one genus and four species, *Savoiaceae* or the Savoy viruses, with one genus and three species, *Lethaceae* or spotted wilt viruses with one genus and one species, a total of sixteen genera and one hundred twenty-eight species.

The *Zoophagineae* include the viruses known to attack animals, particularly insects and mammals. The suborder includes six families, the *Borrelinaceae* or viruses producing disease in insects and other arthropods with two genera and six species, *Borreliotaceae* or the pox viruses, with five genera and twenty-two species, *Erronaceae* or encephalitis viruses with three genera and fifteen species, *Charonaceae* the yellow fever group, with three genera and nineteen species, *Trifuraceae* or infectious anemia viruses, with one genus and two species, and *Rabulaceae* or mumps viruses, with one genus and five species, a total of fifteen genera and sixty-nine species.

Keys to the important families and genera will be found in the Appendix.

CHAPTER 6

The Yeasts, Their Morphology and Classification

Unlike the bacteria, the yeasts do not constitute a major subdivision of the plant kingdom. They are grouped within the great class of *Fungi*. However, certain facts make it convenient to consider them separately. They are unicellular, they have been most satisfactorily studied by the techniques and methods developed in the bacteriological laboratory, they are frequently associated with the bacteria in nature, and, like the bacteria, some produce disease and others bring about fermentative changes of economic importance. They will here be considered as a group, though with the definite recognition that yeasts belong to the fungi and for the most part to the sac fungi or *Ascomycetes* discussed in Chapter 7. Yeasts have sometimes been defined as fungi which do not produce a true mycelium; this definition, while helpful, is not adequate.

MORPHOLOGY OF THE YEASTS

The yeasts, like the bacteria, are predominantly unicellular organisms, but the two groups differ to some degree in the structure, arrangement, and size of their cells, and in methods of sporulation and multiplication. The yeast cell may be ovoid, ellipsoidal, or cylindrical, more rarely spherical or considerably elongated (filamentous). The shape may exhibit considerable variation among individual cells of the same species under different environments.

The *grouping* of the cells shows little of the regularity frequently to be noted among the bacteria. Many yeasts multiply by a type of vegetative budding from any point on the cell. The daughter cells are at first small, but rapidly develop and themselves begin budding. These cells frequently cling together for a time, forming irregular masses. Sometimes the cells are elongate-cylindric and are united in chains or filaments, the budding in such cases for the most part occurring at the tips of the cells. The filaments thus produced may resemble the hyphae (mycelium) of certain molds, and constitute a type of plant structure intermediate between that of the typical yeast and the mold. A few yeasts do not bud, but multiply by fission as do the bacteria. Most yeasts also multiply by spore production.

Size. A few of the smallest yeasts are no larger than some of the bacteria; most kinds are much larger. The greater size of the yeast cell is sufficiently characteristic to be one of the most useful practical criteria in the laboratory differentiation of yeasts from bacteria. The commoner yeasts are usually within the limits of 3–10 μ in diameter by 3–100 μ in length.

Structure—Cell Wall. The yeast cell possesses a relatively firm outer membrane or cell wall composed of a substance sometimes termed yeast cellulose. This wall is apparently absent from the very young cell, but begins to show as a delicate membrane by the time the cell is one-third grown. The chemical composition of the cell wall has not been adequately studied; probably it is closely related to the cell walls of other fungi and is carbohydrate in nature, although it does not give the reactions characteristic of true cellulose. The wall may be considerably thickened in old cells, which may then function as a type of resting cell or spore. Gelatinous capsules of essentially the same character as those found among the bacteria are occasionally present. Flagella are never produced, and the yeast cells are never motile.

Cell Contents. The protoplasm or living contents of the yeast cell may be differentiated into several components. In addition certain cell inclusions are often to be noted, particularly vacuoles, granules, and oil globules. The *ectoplast* in the yeasts, as in the bacteria, is the outer, somewhat differentiated layer of the protoplasm lying just within the cell wall and appressed to it. It probably functions as a semipermeable or osmotic membrane and is an important factor in determining what may enter and what may leave the protoplasm of the cell. A definite *nucleus* in the yeast cell may be demonstrated. It is small relative to the size of the cell and is scarcely to be recognized in the unstained specimen, although proper staining methods may show it distinctly. The nucleus divides preceding the formation of new cells, one-half going to each daughter cell. The nuclear division is a primitive type of mitosis. Evidence in recent years indicates that the process of partition of nuclear material between the mother and daughter cells does not differ essentially from that found in other fungi and the higher plants. The *cytoplasm* of all mature yeast cells is *vacuolate,* that is, it contains spaces filled with cell sap and not with the more viscous protoplasm. Granules of *glycogen* and globules of *oil* may also be found, both probably representing reserve food supply, though the latter may be the result of degenerative processes.

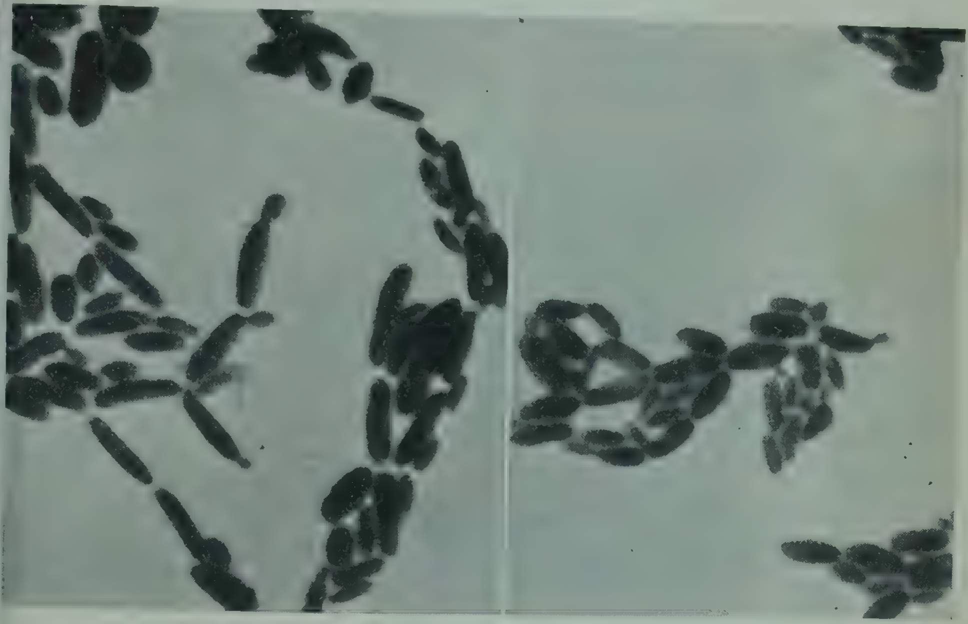

Fig. 6–1. Photomicrographs of yeasts showing budding of the cells. A. Note the elongate cells and a tendency for the buds to form near the ends of the cells. B. Yeast cells showing budding, and some degree of irregularity in staining, due to the presence of vacuoles. It is probable that the cluster of cells has grown from a single cell.

Reproduction of Yeasts. True yeasts reproduce both vegetatively and by means of ascospores. Other yeasts and yeastlike fungi do not produce ascospores.

Vegetative Reproduction. Yeasts reproduce vegetatively in one of two ways, by budding or by fission. In budding a minute protuberance appears on one side of the mother cell, the nucleus divides, and one portion passes into this bud. The bud then enlarges and is separated by a constriction from the mother cell. At first no cell wall is demonstrable, but this soon develops. The daughter cell may separate at once and become entirely free or it may remain attached to the mother cell for a time. This type of multiplication is illustrated in Fig. 6–1.

Multiplication by fission in the genus *Schizosaccharomyces* resembles somewhat the vegetative multiplication in the bacteria. The cell develops to its full size, and a membrane forms across the middle. A dividing cell wall is formed and the two cells separate.

Spore Production. All *true* yeasts produce ascospores and are to be regarded as simple, perhaps primitive, members of that class of fungi called *Ascomycetes* (sac fungi). This group is characterized by the formation of a number of spores within a cell, sometimes one that has been somewhat differentiated for the purpose, though in general the spores develop within cells that are little or not at all different in appearance from the vegetative cells. A cell containing spores is termed an *ascus* or sac, the spores are called *ascospores* (more rarely *endospores*). Asci and ascospores are shown in Fig. 6–2.

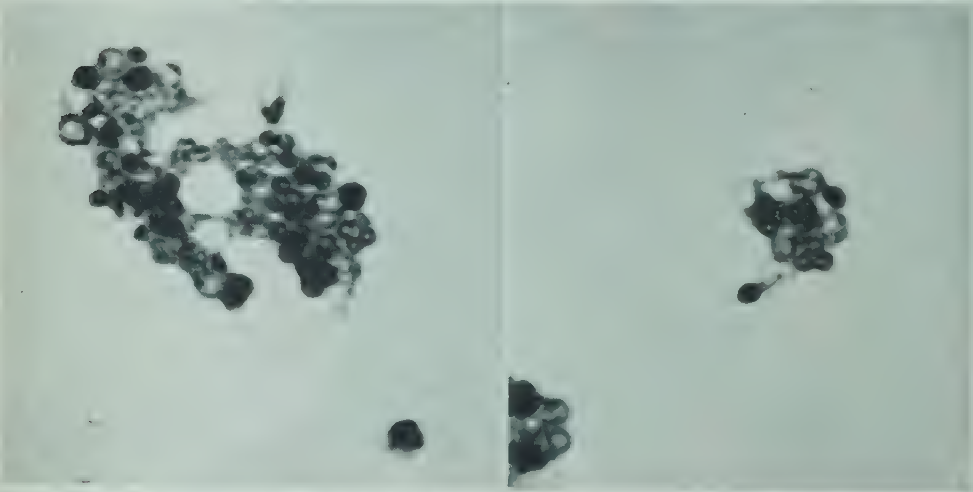

Fig. 6–2. Yeasts showing asci and ascospores. Photomicrographs. The heavily stained cells are still in the vegetative state. Many of the cells have been transformed into asci each containing four spores. In some asci the spores stain heavily (are relatively young), in others lightly (more nearly mature). Some asci are empty; the spores have escaped.

Some yeasts require special conditions for production of ascospores; they are not always to be found in actively growing cultures in nutrient solutions. E. Chr. Hansen formulated the following general rules for inducing spore production in some of the common yeasts. (1) The cells must come from a young, well-nourished culture. (2) The culture must be well aerated. (3) There must be an abundance of moisture. (4) The temperature must be somewhat high, the optimum for most types being about 25° C. These conditions are met by the use of a sterile block of plaster of paris set in sterile water with the upper surface exposed to the air. The top is seeded with yeast from a vigorous, young, well-nourished culture and placed at 25° C. for twenty-four to forty-eight hours. Usually the spores will have developed in this time. Spores may sometimes be found in films of yeasts floating on nutrient solutions and are produced readily by some yeasts when grown on solid media.

Usually a constant number of spores will develop in the asci of a given species. Some yeasts develop asci with one spore only; usually there are from two to four, sometimes eight. The spores are formed by a division of the nucleus into a number of nuclei corresponding with the number of the spores to be developed. Each daughter nucleus becomes surrounded by a portion of the cytoplasm, and this in turn by a spore membrane. Sometimes the ascus or mother cell disintegrates after the formation of the spores; usually, however, the old wall is relatively persistent. The spores may be spherical, ellipsoidal, kidney-shaped, spindle-shaped, or flattened on one side. They may be smooth or banded by lines or rings.

Spore formation in true yeasts, as generally in the sac fungi (*Ascomycetes*), can best be interpreted on the basis of a life cycle in the yeast in which there occurs at intervals the fusion of two nuclei, giving rise to a nucleus having a doubled number of chromosomes. Usually, when this phenomenon of fusion of two nuclei occurs, two cells which were originally separate, unite. Sometimes, however, two nuclei may fuse after a nuclear division within the cell, or there may be a fusion between the nucleus of the mother cell and that of a daughter cell. In any case, the nucleus as a result of the fusion has double ($2N$) the previous number (N) of chromosomes. In general, any cell in which the nucleus has a double complement of chromosomes is said to be diploid.[1] A cell in which the nucleus contains a single complement of chromosomes is termed *haploid*.[2] The cell in which the fusion of two nuclei occurs, with the development of the haploid nucleus, is called a *zygote*.[3] In general in the nuclear divisions which immediately precede and are a part of spore formation, there is one division in which the number of chromosomes going to each daughter nucleus is one-half that of the diploid. Each spore therefore is a haploid; it contains the single complement of chromosomes.

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[1] From the Greek word *diplous*, meaning doubled.

[2] From the Greek *haplous*, meaning single.

[3] From the Greek word *zygotus*, meaning joined together, united or yoked.

Different genera and species of yeasts show differences in the life history, depending upon the sequence of formation of diploid and of haploid cells. The following general types of life cycles have been shown to occur.

Type 1. The diploid nucleus of the zygote divides into two nuclei, each having the diploid number of chromosomes, and the daughter cells repeat the process indefinitely. The yeast cells derived from the zygote, and the progeny of these cells are therefore all diploid; this period of cell multiplication has been termed, therefore, the diploid phase. This series of diploid generations continues until there is the stimulus for spore formation, whereupon the nucleus produces daughter nuclei having the haploid number of chromosomes, each forming an ascospore all within the mother cell which becomes a sac or ascus. Under favorable conditions these spores germinate, each becomes a yeast cell with a haploid nucleus. The daughter cells all have haploid nuclei. Again, this multiplication of haploid cells may continue indefinitely. Under proper stimulus eventually some of the cells fuse in pairs, the nuclei unite to form a diploid nucleus in the zygote, and a cycle has been completed. The life cycle of this type of yeasts may be represented diagrammatically as follows:

> Zygote (diploid) → yeast cell (diploid) multiplication (all cells diploid) → ascus formation with haploid spores → spore germination to haploid yeast cell → multiplication (all cells haploid) → cell fusion and formation of zygote (diploid).

Or even more simply:

> Zygote → diploid multiplication phase → ascus and spore formation → haploid multiplication phase → cell fusion and formation of diploid zygote.

The cells which fuse to form the zygote are termed *gametes*, and the fusion is regarded as a simple sexual process.

Type 2. This type differs from Type 1 above in that there is almost complete elimination of the haploid multiplication phase. The spores of the ascus fuse in pairs, and the vegetative cells are consistently diploid. This may be illustrated.

> A diploid cell becomes an ascus with haploid spores → the spores act as gametes, fusing in pairs with production of diploid nuclei → zygote → diploid vegetative multiplication → ascus

Type 3. This type differs from Type 1 in that there is practical elimination of the diploid multiplication phase. The vegetative phase is essentially haploid. Upon fusion of cells to form a zygote there is immediate formation of an ascus with haploid ascospores which in turn become vegetative haploid cells. It may be illustrated.

> Zygote (diploid) → ascus with haploid spores → vegetative haploid cells → gametes and zygote.

There may be variations from the three types listed above. The yeast genera are differentiated largely upon the bases of the type of life cycle shown, and morphology of cells, ascus and spores, and to some extent upon physiological, particularly fermentation, characters.

True and False Yeasts. The yeasts do not constitute a homogeneous group. Among the several great groups of fungi there may be found organisms which can adapt themselves to growth in a liquid medium in which the cells increase by budding. Even fungi such as the corn smut which normally produce a mycelium may reproduce in this fashion indefinitely under appropriate conditions. Usually, however, the term yeast is restricted to those organisms which show throughout their life history little or no tendency to develop a true fungal mycelium (see Chapter 7).

Some of the yeasts evidently are simple members of the *Ascomycetes* or sac fungi and have the ability under suitable conditions to produce *asci* and *ascospores*. These are often termed the *true yeasts,* sometimes the *sporogenous* yeasts, *ascosporogenous,* or *ascogenous* yeasts. Inasmuch as other yeasts or yeastlike fungi may produce *spores* but not *ascospores* or *asci,* the terms *ascogenous* or *ascosporogenous* are preferable. A large proportion of the yeasts producing economic fermentations belong to this group.

There are also many yeasts and yeastlike fungi which reproduce vegetatively like yeasts but are not known to produce ascospores. These are sometimes grouped together as the false yeasts and placed by the mycologists with the imperfect fungi (see Chapter 7). They are also technically known as the *anascosporogenous* or *anascogenous* yeasts.

CLASSIFICATION OF THE YEASTS

The heterogeneous group called the yeasts may be divided into three groups on the basis of apparent or assumed relationships, those which produce asci and ascospores and evidently belong with the

Ascomycetes or sac fungi, those which produce spores on special pointed projections from the cells (from *basidia*) and are apparently primitive *Basidiomycetes*, and those which produce neither basidia nor asci, the false yeasts, placed sometimes with the imperfect fungi (*Fungi Imperfecti*). The five families commonly recognized may be differentiated by the key in the Appendix. These families are the *Saccharomycetaceae*, *Rhodotorulaceae*, *Cryptococcaceae*, *Nectaromycetaceae*, and *Sporobolomycetaceae*.

Saccharomycetaceae.[1] This family includes the so-called true yeasts, and most of the species which are of industrial importance. It is subdivided into four subfamilies and fifteen or more genera. Keys may be found in the Appendix. A few only of the more important genera will be described.

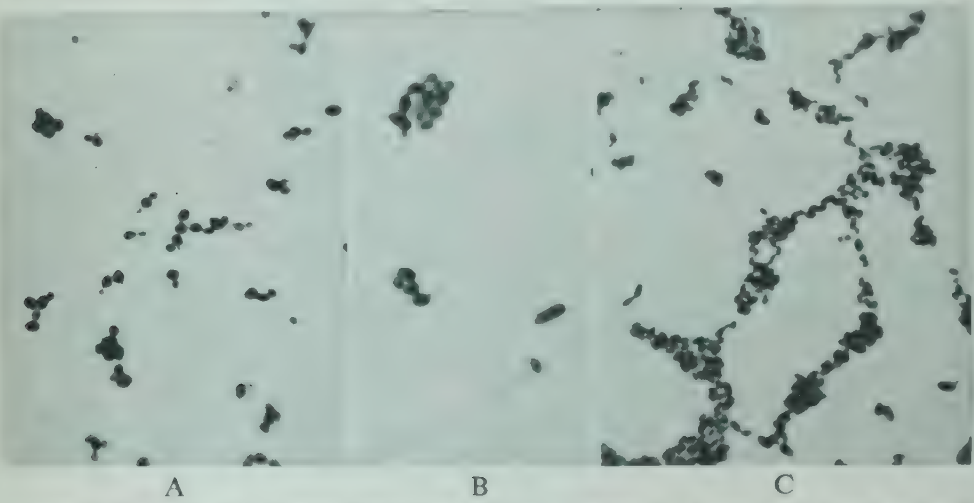

Fig. 6–3. Yeasts. Photomicrographs. A. Cells nearly spherical, showing bud formation on many sides of the cells. B. Cells larger, spherical to ellipsoidal. C. Cells relatively small, ellipsoidal.

The genera of this family are not always readily differentiated. Morphological characters are used as far as possible, but must in some cases be supplemented by physiological characters. The form of the vegetative yeast cell may be distinctive. In some species the cells may be spherical, oval, ellipsoid, even elongate and cylindrical. The cell shape may vary with the medium.

The method of vegetative multiplication is significant. Most yeasts multiply by budding as has been described, some by transverse fission, and some use both methods, or something intermediate. Those which produce a mycelium apparently form cross walls in much the same manner as do the molds.

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[1] Sometimes called *Endomycetaceae*. The name *Saccharomycetaceae* is older and preferable.

In some yeasts which produce a mycelium, buds may form which function as spores, so-called *blastospores* or *conidia*. Some yeasts may also produce *oidia* or *arthrospores* which are formed by segmentation of a chain of cells.

As has been noted above, production of ascospores is frequently (usually) preceded by a process of sexual fusion of two cells, followed immediately or after a series of cell divisions by the formation of one to eight (usually four) cells or spores. A few yeasts are known in which fusion of cells (gametes) does not occur, and the ascospore (frequently only one) formed in the ascus does not differ in chromosome number from the mother cell.

Yeasts differ with respect to the time in the life history when cell fusion occurs and in consequence as to whether the vegetative cells are haploid or diploid.

A further complication in life history of some of the yeasts arises from the fact that the two cells (or *gametes*) which fuse in some forms are apparently of the same size and the cell fusion is said to be *isogamous;* in other forms the gametes are of two sizes, large and small, and copulation (cell union) occurs only between a small and a large cell, and the yeast is termed *heterogamous*.

In some isogamous yeasts, cell fusion does not occur unless the cells are of different strains. For example, it was found that the descendants of a single ascospore would not fuse with each other, but with the descendants of another spore. In other words, each ascus produces spores of two kinds, and the vegetative cells originating from these are also of two kinds, in appearance the same, but one potentially a *male* or *plus* strain and the other a *female* or *minus* strain. Cells of the two different haploid strains may fuse. In modern genetic studies, it is assumed that the genes which control the heredity of the cell are located in the chromosomes. Because of their rapidity of multiplication, and the ease with which they may be crossed, geneticists have found the yeasts excellent material for studies of problems of heredity.

A few of the fifteen or more genera of yeasts will be noted briefly.

Saccharomyces is the largest and most important of the yeast genera. The vegetative cells are usually diploid, conjugation occurring between ascospores within the ascus or soon after its rupture. Stelling-Dekker recognized twenty-four species; the varieties and strains are legion. The yeasts most commonly associated with alcoholic fermentation in brewing, breadmaking, wine making, in distilleries, and in production of commercial alcohol belong here. Because of their great economic importance these yeasts have been much studied, and various methods of classification have been developed. One of these methods of classification was based upon the shape of the cells. To the common bread yeast having large spherical or ovoid cells the name *Saccharomyces cerevisiae* was given (Fig. 6–4A), to the wine and cider yeasts having ellipsoidal or short oval cells the name *Sacch. ellipsoideus* (Fig. 6–4B), and to all long or cylindric-celled yeasts *Sacch. pastorianus* (Fig. 6–4C). The extreme variability in morphology of the same yeast under different cultural conditions renders an application of such a classification difficult. These names, however, are very commonly used to express the general morphologic type under consideration. Another classification used extensively in the brewing industry is to differentiate between "bottom" and "top" yeasts. In some types of yeasts, the cells remain almost wholly below the surface of the fermenting liquid during the process of fermentation, forming a sediment on the bottom; hence the name *bottom yeast*. In others, the organisms form a frothy scum on the surface during the process of active fermentation, and only after fermentation has largely ceased does this superficial mass break to pieces and settle to the bottom. To this type the name *top yeast* is applied. Hansen has shown, however, that these characteristics are not constant and concludes that they do not constitute a satisfactory basis for classification. Physiologic characters, such as ability to ferment various sugars, and cultural characters on solid media have in recent years been given greater prominence in the formulation of classifications.

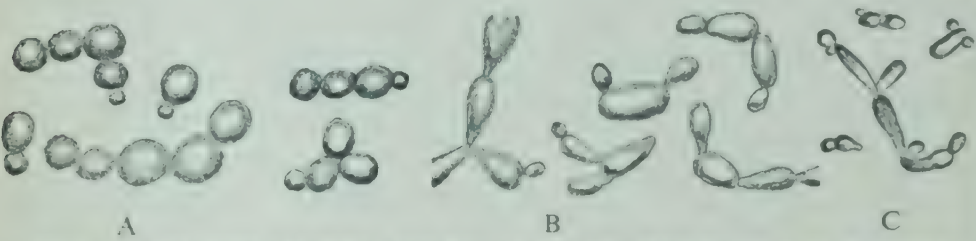

Fig. 6–4. Drawings of cells of some species of the yeast genus *Saccharomyces*. A. *Saccharomyces cerevisiae*, the bread and beer yeast. B. *Saccharomyces ellipsoideus* one of the wine yeasts. C. *Saccharomyces pastorianus*. (All adapted from Hansen.)

The species of the genus *Saccharomyces* are separated into numerous subgroups and species on the basis of their ability or lack of ability to ferment various sugars with production of alcohol and gas. All the commonly recognized species ferment the three monosaccharides, dextrose, levulose, and mannose. Dekker recognizes ten subgroups on the basis of fermentation of sugars. Four other sugars, the monosaccharide galactose, the disaccharides sucrose and maltose, and the trisaccharide raffinose are used in differentiation.

Key to the Subgroups of the Genus *Saccharomyces*

a. Galactose + .
  b. Sucrose — . Group 1 (3 species).
  2b. Sucrose + .
    c. Lactose — .
      d. Maltose — . Group 4 (2 species).
      2d. Maltose + .
        e. Raffinose — . Group 7 (1 species).
        2e. Raffinose + .
          f. Raffinose ⅓ fermented. Melibiose — . Group 8 (6 species).
          2f. Raffinose completely fermented. Melibiose + . Group 9 (5 species).
    2c. Lactose + . Group 10 (1 species).
2a. Galactose — .
  b. Maltose — .
    c. Raffinose ⅓ fermented. Melibiose — . Group 2 (2 species).
    2c. Raffinose completely fermented. Melibiose + . Group 3 (1 species).
  2b. Maltose + .
    c. Raffinose — . Group 5 (1 species).
    2c. Raffinose + , ⅓ fermented. Melibiose — . Group 6 (2 species).

Raffinose is a sugar which may be split into the monosaccharide fructose and the disaccharide melibiose. Those yeasts which ferment one third of raffinose apparently ferment the fructose portion, leaving the melibiose fraction unchanged. Other yeasts ferment the raffinose completely; they ferment not only the fructose but also the melibiose. If there is doubt as to the ability of a yeast to ferment raffinose in part or completely, the doubt can be removed by determining ability to ferment melibiose. A key to twenty-four species of *Saccharomyces* is given in the Appendix.

Zygosaccharomyces. This genus is differentiated from the next by the fact that at least some of the cells fuse in pairs before spore formation, representing a primitive type of sexual reproduction. Spores form not only on gypsum blocks but also on solid media. Of the fifteen species described none is of much economic importance. Some are active in producing fermentation.

Schizosaccharomyces. This genus is easily differentiated because of its vegetative multiplication of cells by fission and not by budding. Two species have been described.

Other genera of *Saccharomycetaceae.* Altogether about fifteen genera of yeasts are recognized. Keys for their separation will be found in the Appendix.

Rhodotorulaceae. This family of yeasts is characterized by the presence of a reddish pink, orange, or even yellow pigment, and by lack of production of asci and ascospores. One genus only is recognized, *Rhodotorula.*

The genus *Rhodotorula* includes many so-called species which are not too well differentiated. Some authors suggest that there is but one highly variable species, *Rhodotorula glutinis.* The pink yeasts are not of much economic significance. They are not uncommon in dust, and they occur most frequently in acid fermenting materials such as sauerkraut. They are sometimes found in wines and in dairy products. The monograph by Lodder lists thirteen species differentiated on such characteristics as ability to utilize nitrates as a source of nitrogen, shape of cells, color and sliminess of growth in culture.

Cryptococcaceae (or **Torulopsidaceae**).[5] This yeast family includes those yeasts that do not produce ascospores, do not produce conidia, and do not contain a red or orange (carotinoid) coloring matter. They constitute a group of considerable economic importance; some of them are of significance in fermentations, some are parasitic, and some are able to produce disease in animals and man.

The family is divided into two subfamilies, primarily on the development of a so-called pseudomycelium.

In one subfamily, the *Cryptococcoideae* (or *Torulopsidoideae*), multiplication is by budding, without the formation of long cells in chains and without the development of spores (blastospores) at the nodes. The second family, the *Mycotoruloideae*, differs in that there are produced a pseudomycelium and asexual spores.

The subfamily *Cryptococcoideae* (or *Torulopsidoideae*). The yeasts of this subfamily in many respects resemble those of the true yeasts, but differ primarily in that they do not produce asci and ascospores. They multiply by budding and do not form a pseudomycelium or mycelium. Probably some of the species are true yeasts that no longer are able to produce ascospores. Perhaps in some cases they represent a haploid strain which has no counterpart with which to conjugate.

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[5] Three names, *Cryptococcaceae*, *Torulopsidaceae*, and *Torulopsaceae*, are currently used for this family of yeasts. Which is to be finally adopted will probably be determined eventually by international agreement.

The seven genera known are differentiated from each other on the basis of cell shape, method of budding, and to some extent upon cultural and physiological characteristics. Several genera are of some economic significance.

The genus *Torulopsis* (or *Cryptococcus*) is perhaps the most important. Lodder recognizes twenty-two species, of which eleven resemble true yeasts in ability to ferment sugars (at least dextrose) with production of carbon dioxide and alcohol, and which may be important in alcoholic fermentation, as for example *Torulopsis kefyr* and *T. sphaerica* in fermentation of milk, *T. pulcherrima* in fermentation of fruit juices, and *T. utilis* in production of yeast cake to be used as a food. To the non-fermentative eleven species the generic name *Cryptococcus* is often applied. Many of these species are parasitic; some, as *Torulopsis neoformans,* are capable of producing disease in man.

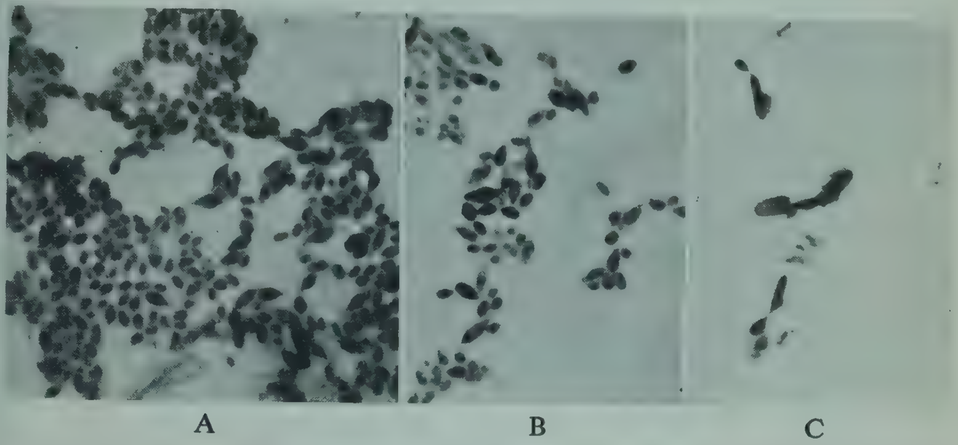

Fig. 6–5. Yeasts. Photomicrographs. A. Note the slender projections from one or both ends of many of the cells, also the vacuolate cell contents. B. Some cells are flask shaped, the buds are formed at the end of the stalks. C. Note the slender stalk connecting the bud to the mother cells.

The species of the genus *Mycoderma* are differentiated from those of *Torulopsis* in part by the prompt production of a dry film of growth on beer wort; they do not produce alcoholic fermentation. These yeasts form films on the top of fruit juices, etc., undergoing alcoholic fermentations produced by other organisms, and oxidize the alcohol formed.

The members of the yeast genus *Kloeckera* are lemon-shaped. All are able to ferment sugars. The cells in *Trigonopsis* tend to be triangular, with budding occurring at the corners.

The yeasts of the subfamily *Mycotoruloideae* (*Candidoideae*) are characterized by the formation of chains of cells which resemble the mycelium of the true molds. From the nodes (joints) of the cell, cells (blastospores) are budded off. In one genus (*Trichosporon*), the hyphal threads, particularly near the ends, also tend to separate by formation of cross walls with formation thereby of chains of spores termed *arthrospores*. The genus *Brettanomyces* has cells which are usually round, oval, or even elongate, with the end of the cell having the shape of a pointed arch. Under aerobic conditions the organisms produce considerable acidity. The pseudomycelium with blastospores is poorly developed. The four species have been isolated from beer. All actively ferment certain sugars with production of alcohol and CO_2.

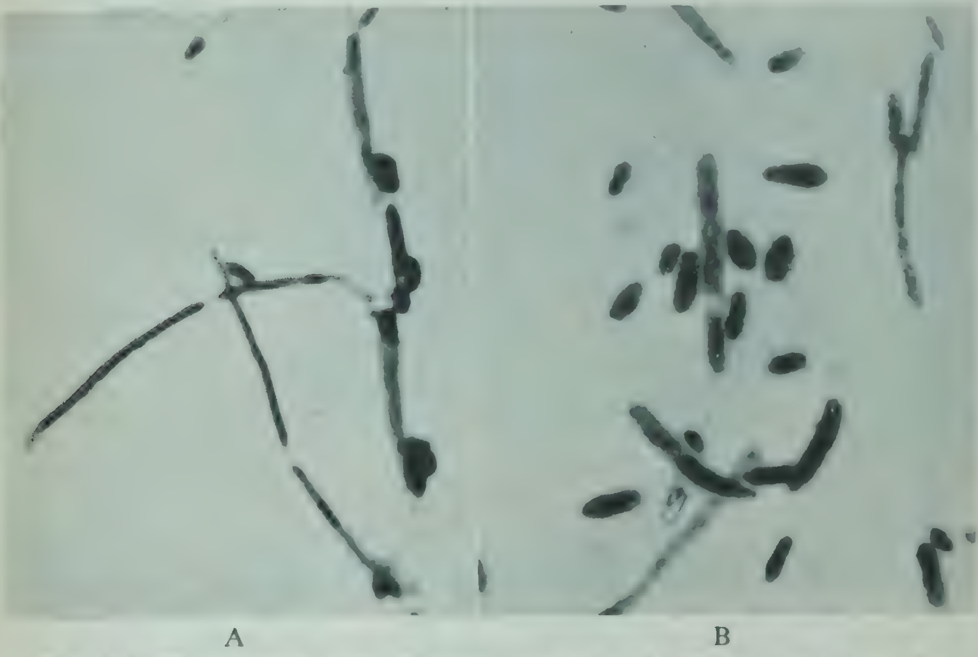
A B

Fig. 6–6. Filamentous yeasts. Photomicrographs. A. Filamentous yeast showing branching of the pseudomycelium. B. Filamentous yeast in which the mycelium has fragmented, and in which numerous smaller cells have budded off, to function as spores.

The genus *Candida* is a large one; Diddens and Lodder recognize twenty-eight species. The pseudomycelium is well developed, and blastospores are formed at the joints, sometimes in large numbers. Several species are associated with disease, others are found in fermentation. This group of yeasts has been much studied. Many of the species were formerly but incorrectly included in the genus *Monilia*. To illustrate the complexity of the problems of nomenclature in this group, it may be noted that the commonest pathogenic species, *Candida albicans,* according to Diddens and Lodder has eighty-eight synonyms.

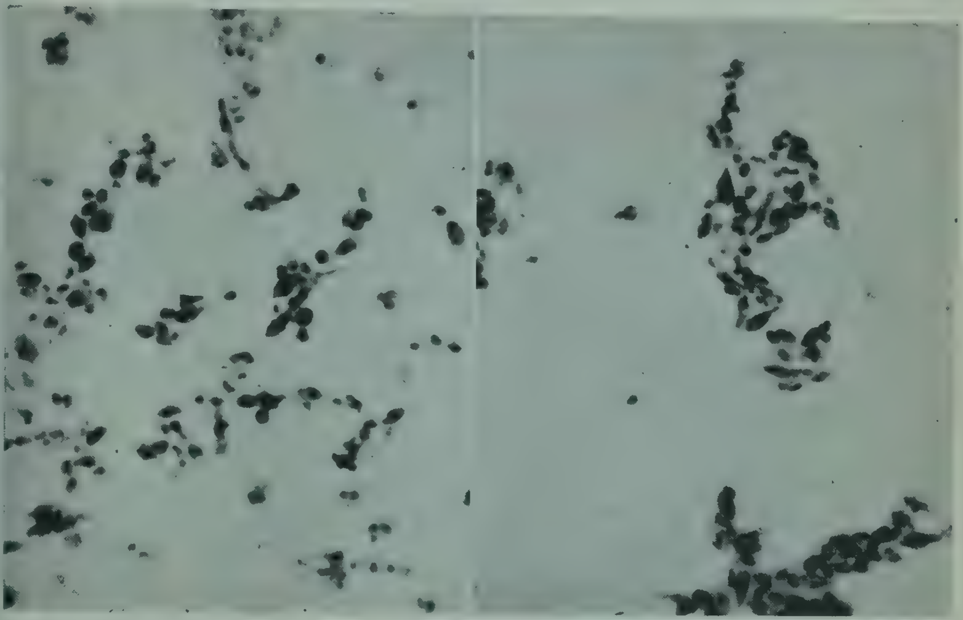

Fig. 6–7. Autolysis of yeasts. Photomicrographs. Note that most of the cells are highly vacuolate, some seem to be completely free of protoplasm; ghost cells or shadow cells are evident.

CHAPTER 7

The Molds, Their Morphology and Classification

Like the yeasts, the molds belong to that great division of plants called the *Fungi*. The molds constitute a group which has no real systematic standing; they are distributed among the various subdivisions of the fungi; they are considered together largely because of their special economic significance. They are found in nature in the same places as are the bacteria and the yeasts, and in some cases produce fermentations and other changes as do yeasts and bacteria. They may be studied by the same techniques in the laboratory.

The molds are multicellular. In this they differ from the unicellular yeasts and bacteria. This type of plant body renders the discussion of morphology more complex than with bacteria and yeasts, for it is necessary to consider not only the individual cells, but also how they are combined to make up the mold body and how they are differentiated into the several types of hyphae found in the various mold organs. Several hundred genera of molds have been described, contrasting with the somewhat smaller number among the bacteria and the yeasts.

MORPHOLOGY OF THE MOLDS

Plant Body of the Mold. Molds, as well as most other fungi, are made up of more or less branched threads or *hyphae*. These hyphae are differentiated on the basis of function into two types: some serve to secure food from the material (substrate) in which they are growing, and are called *vegetative hyphae;* others may produce spores and are said to be *fertile hyphae*. The whole mass of vegetative hyphae, the entire plant body of the mold, is called the *mycelium*. Molds may be differentiated into two types on the basis of the kind of vegetative hyphae each produces. The hyphae in the one case are *septate*, in the other *non-septate*. A septate hypha is divided at intervals by cross walls or *septa* (sing., *septum*), that is, the thread is divided into distinct compartments. In the *non-septate* type, cross walls are wholly absent or occur only in fertile hyphae and in formation of spores or of spore-bearing structures; in such molds the whole mycelium is made up of a more or less branched continuous tube containing the protoplasm. In molds in which the hyphae are septate, each cell contains one or more nuclei. The division of the nucleus (or nuclei) is regularly followed by the formation of a cross wall or septum separating the two daughter cells. In some cases the cell regularly contains two nuclei. In molds with non-septate hyphae there are several to many nuclei; such a hypha is sometimes termed a *coenocyte*.[1] Whether or not the mycelium of a mold is coenocytic or septate is a useful criterion in classification, i.e., two of the principal groups of molds may be differentiated on this basis.

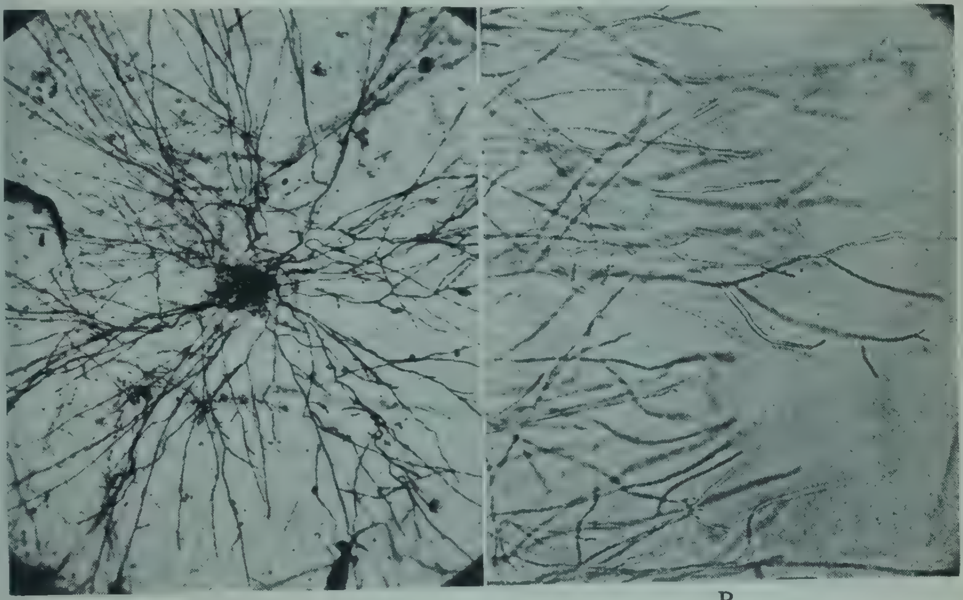

Fig. 7–1. Mold mycelia. A. A young mold colony showing the radiating hyphae and the mode of branching. B. The margin of a colony of *Aspergillus* showing the branching vegetative hyphae as they grow through the nutrient medium.

The structure of the cells of a hypha does not differ materially from that of yeast cells. There is present the cell wall and the protoplasm made up of ectoplast, cytoplasm, and nucleus or nuclei. The cells frequently contain vacuoles, granules, oil globules, and sometimes other inclusions. The cell wall is made up in some instances of a substance that gives the reactions characteristic of the cellulose in higher plants; most frequently, however, it consists of a nitrogenous material resembling chitin. These walls may resemble in composition the cell walls of the bacteria.

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[1] Or a *syncytium*.

Growth of a Mold. All multicellular organisms grow in two ways, by an increase in size of the component cells and by an increase in number of these cells. The increase in number of cells in a mold hypha may be either *intercalary* or *apical*. In molds showing the intercalary division any cell of the hypha may divide, each cell then increasing in size, frequently with some consequent distortion of the thread. Such intercalary multiplication of cells is relatively rare. In apical growth, by far the commoner type, the terminal cell alone divides, and the filament grows in length only at and near its tip. Branching of a hypha is due to the budding out of a lateral filament from a cell.

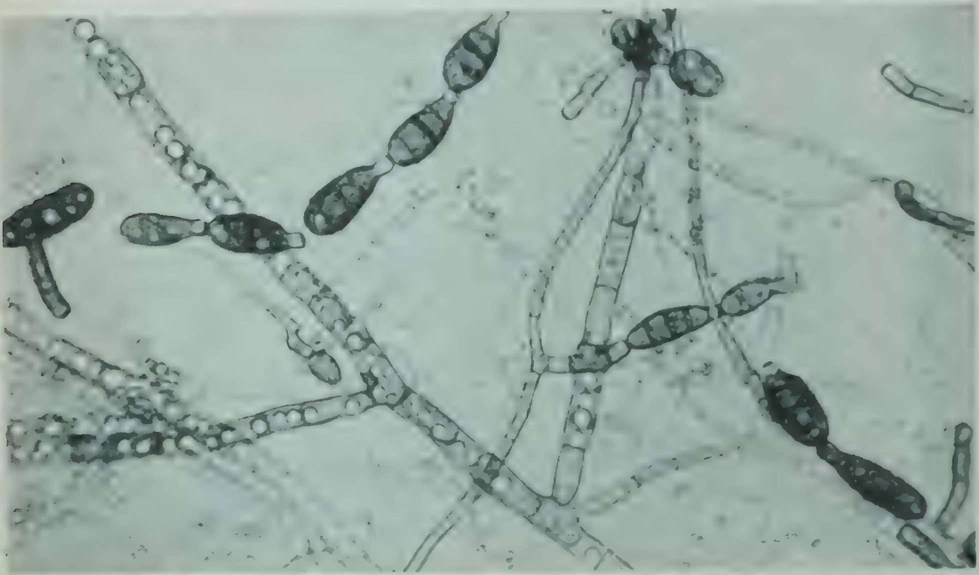

Fig. 7–2. Mycelium and spores of the mold *Alternaria*. Note the septate mycelium, the branching of the hyphae, and the presence of fat globules in the larger and older cells. The slender hypha near the center is a conidiophore, with a chain of spores developing from a side branch. Above, a chain of dark, multiseptate conidia.

Reproduction of Molds. Molds reproduce usually by means of spores, each organism frequently producing these spores in great numbers. Spore production by molds, therefore, unlike that by bacteria, is a method of *multiplication* as well as of *reproduction*. A spore may be defined as a more or less differentiated cell or group of cells destined for the reproduction of the organism. Like those of bacteria and yeasts, the spores of the molds are useful in carrying the organism over unfavorable conditions in that they are somewhat more resistant to drying. Frequently they are blown about, carried by insects, or even forcibly expelled by the fungus.

Mold spores may be grouped into two principal categories, they are sexual or asexual in origin. A *sexual spore* is one that is either the immediate or the indirect product of the fusion of two cells, called *sex cells* or *gametes*. An *asexual spore* is one that is produced without the intermediation of sex cells. Practically all molds produce asexual spores, frequently more than one kind; many molds produce sexual spores as well.

In practical work in the laboratory the molds as studied usually are found to produce asexual spores, and identification must frequently be made without the opportunity to observe sexual spores. Many groups of molds are not known to produce sexual spores. However, the basic botanical classification of the molds as fungi is outlined as far as possible upon the characteristics of the sexual spores.

Sexual Spores of Molds. All the fungi, including the molds are usually divided into four great classes, three on the basis of the type of sexual reproduction, and one on the basis of lack of production of sexual spores. The three principal types of sexual spores are zygospores, ascospores, and basidiospores.

The zygospore of *Mucor* may serve as an illustration of this type of spore formation. Two cells approximately equal in size arise from filaments lying close together, enlarge, and bend toward each other. Their tips are separated from the remainder of the branch by the formation of cell walls. Finally the tips meet, the walls in contact dissolve, and the contents of the two cells flow together and fuse to form the *zygospore* (yoke spore). A heavy membrane is laid down about this cell which then goes into a resting stage. Under favorable growth conditions it will germinate and reproduce the mold. In some species two types (usually termed + and − strains) of mycelium are known, not differentiable on the basis of morphology, and neither alone capable of producing zygospores. When plus and minus strains are grown side by side many zygospores are produced where the mycelia of the two are in contact.

Many molds having septate mycelia are known to produce *ascospores* at some time during the life cycle; probably others will be shown to produce them also when their complete life cycles have been studied. This type of spore formation may be illustrated by

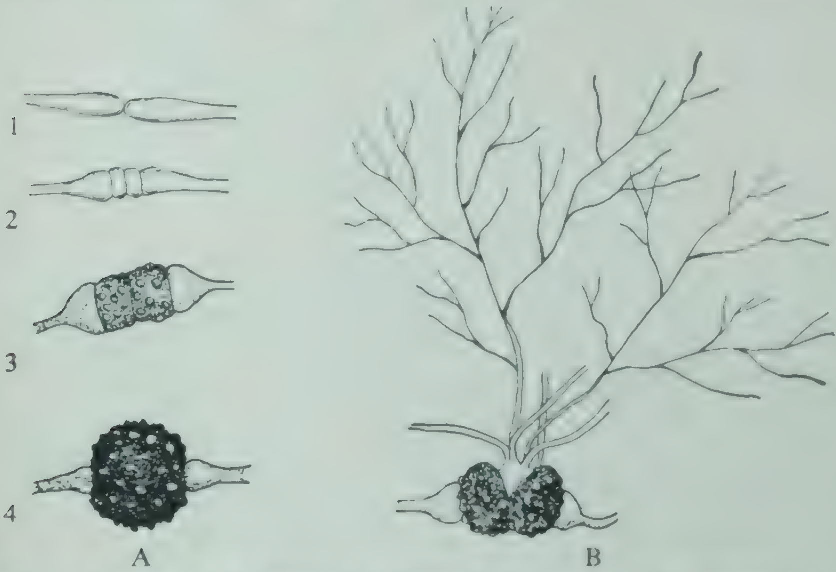

Fig. 7–3. Zygospore of *Mucor*. A. Stages in formation of zygospore. 1. Two hyphae from distinct strains of *Mucor* approaching each other. 2. The terminal cells have been walled off and are in contact. 3. Cells fusing and enlarging. 4. Ripe zygospore with suspensors on each side. (Adapted from Brefeld.) B. Germination of the zygospore. (Adapted from Tavel.)

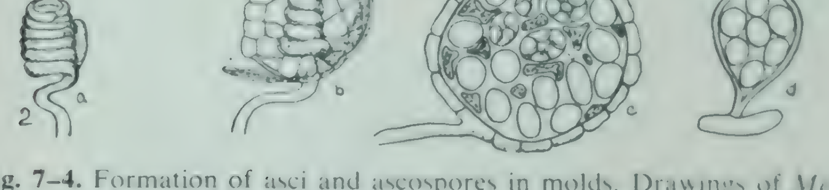

Fig. 7–4. Formation of asci and ascospores in molds. Drawings of *Monascus* and *Aspergillus*. 1. *Monascus: a.* Sex cells branching from hyphae. *b, c.* Formation of the perithecium by the growth of sterile hyphae to form a covering. *d.* Section through a mature perithecium showing the ascospores which have escaped from the asci and are lying free within the perithecium. 2. *Aspergillus: a, b, c.* Stages in the development of the perithecium; ascus with ascospores from the interior of the perithecium. (1, adapted in part from Harz, 2, adapted from de Bary.)

Aspergillus. Two cells, of the same or different sizes, arise on the same or on adjoining hyphae. They coil together, the tips fuse, and the contents of one cell pass to the other. Instead of developing immediately into a spore, this fertilized cell grows into a more or less dense mass of branching threads, certain cells of which develop into *spore sacs* or *asci*. In each one of these asci there is produced from two to many spores, the usual number being eight. Frequently the hyphae lying near the sex cells branch and rebranch to form a covering for the mass of asci. Such a structure is called a *perithecium*. A few of the genera of molds commonly encountered produce such perithecia, the principal one being *Aspergillus*. The botanist makes considerable use of perithecia and their contents in formulating logical classifications of the fungi, but because of their rarity they are of little value in the separation of many forms that are of great economic importance. Strain (plus and minus) differentiation of individual mold plants is sometimes observed, perithecia being formed only where two colonies of different strains come in contact.

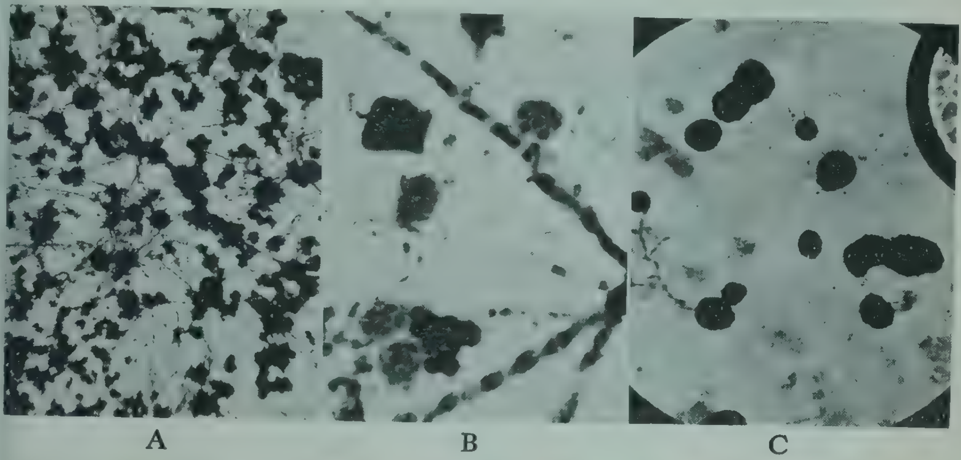

Fig. 7–5. Perithecia and ascospores. A. Hyphae and hairy perithecia of a mold. B. Several perithecia have been crushed and the asci each with eight ascospores released. C. Relatively smooth perithecia of another species.

Some of the *Ascomycetes* have a much more complicated life history than the *Aspergillus* used as an illustration. Many of the fungi of this group show a marked tendency for the intermingling of cell contents when two cells, particularly two cells not closely related, come into contact. The cell walls dissolve at the point of contact, and the nucleus of one cell passes into the other. However, the two nuclei remain separate, and usually when the cell divides both nuclei furnish a daughter nucleus to the new cell. Each cell of succeeding generations contains two nuclei, and the cell is termed a *dicaryon*. In some forms the situation is made even more complex by the presence of numerous nuclei in each cell. Organisms of these types have proved to be of great value in the study of certain genetic problems, but the study of their life cycles constitutes a phase of the science of *mycology*, rather than of microbiology.

The third type of sexual reproduction among the fungi culminates in the formation of basidiospores. Very few of these fungi are classified among the molds. Common examples of fungi producing basidiospores are the mushrooms, toadstools, puffballs, rusts, and smuts. In these fungi the cells of the hyphae are usually dicaryotic, each cell containing two nuclei. These two nuclei have come from an earlier fusion of two cells. In most forms the hyphae are distinguished by the presence of so-called clamp connections. When the cell divides, each nucleus divides, the daughter nucleus of one of the nuclei (nucleus A) passes to the new cell, and the partition wall forms. A short tube is formed around the new cell wall at one side connecting the two cells; one of the daughter nuclei of nucleus B passes through this connecting tube to the other cell. When spores are to be formed, as in a mushroom, the two nuclei fuse in the tip cell of a hypha.

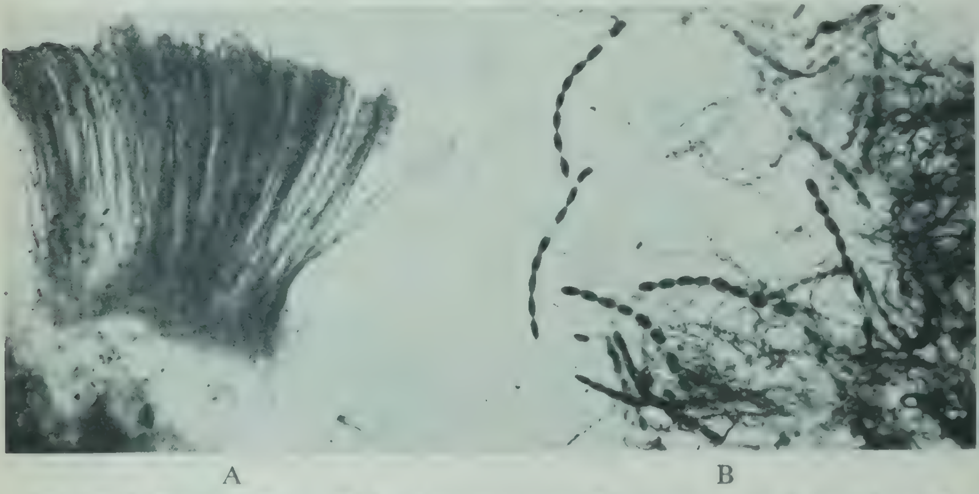

Fig. 7–6. *Peziza*. One of the *Ascomycetes*. Photomicrograph to show the formation of the asci and the ascospores. A. A mass of parallel elongate asci with sterile filaments or paraphyses between. Note the colorless spores. B. Stained preparation showing the elongate asci and the eight spores per ascus. The slender sterile threads or paraphyses lying between the asci are seen to be granular.

which then swells up to form a clublike cell called a *basidium*. The nucleus divides, and (usually) four nucleated spores are produced, one each on the tips of four small, usually pointed projections called *sterigmata*. A few molds show clamp connections, or exhibit a tendency to produce spores in fours (rarely in pairs) on sterigmata, and are placed therefore in the *Basidiomycetes*.

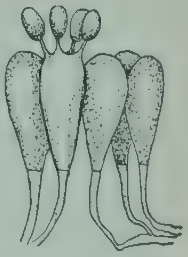

Fig. 7–7. A portion of the hymenium (outer layer of a gill) of a mushroom, showing a cluster of basidia and spores typical of a *Basidiomycete* with the four terminal spores, borne on sterigmata.

Asexual Spores of Molds. Practically all the molds bear asexual spores. The manner in which these are produced, their size, shape, color, and septation are important guides in the determination and differentiation of genera and species.

Asexual spores are either borne free on the sides or ends of hyphal threads, or they are produced in a specialized spore case called a *sporangium*. A spore not borne in a sporangium is commonly called a *conidium*. A few molds are able to produce two or even more kinds of spores, rendering the problem of classification somewhat difficult.

In some molds spores are produced merely by the segmentation of the mycelium, the hyphae breaking up into conidia called *arthro-*

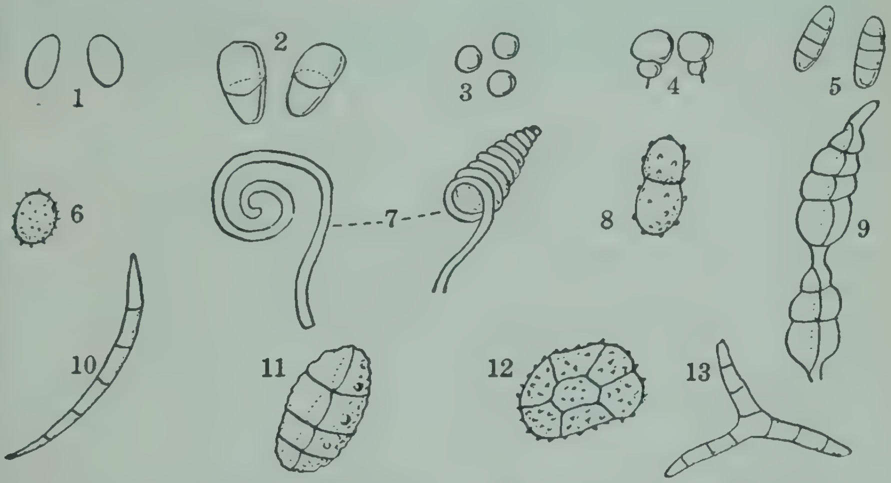

Fig. 7–8. Different types of conidia. 1, *Oöspora;* 2, *Trichothecium;* 3, *Aspergillus;* 4, *Mycogone;* 5, *Septocylindrium;* 6, *Oedocephalum;* 7. *Helicomyces;* 8, *Trichocladium;* 9, *Alternaria;* 10, *Fusarium;* 11, *Macrosporium;* 12, *Epicoccum:* 13, *Trinacrium.*

spores (sometimes termed *oidia*). In some cases spores called *chlamydospores* are formed directly from the cells of the mycelium by enlargement and particularly by the formation of a heavy wall. These resemble arthrospores in that they are formed directly by transformation of a hyphal cell into a spore; they differ, however, by the possession of a heavy membrane and in that the entire hypha is not broken up into spores. In some other molds the conidia are produced directly on the sides or ends of hyphae which differ in no way from vegetative hyphae. Usually, however, the conidia are borne on specially differentiated fertile hyphae. A stalk of this type bearing conidia is termed a *conidiophore,* one bearing a sporangium a *sporangiophore.* The grouping, size, shape, and branching of the conidiophores and sporangiophores are important in the differentiation of genera.

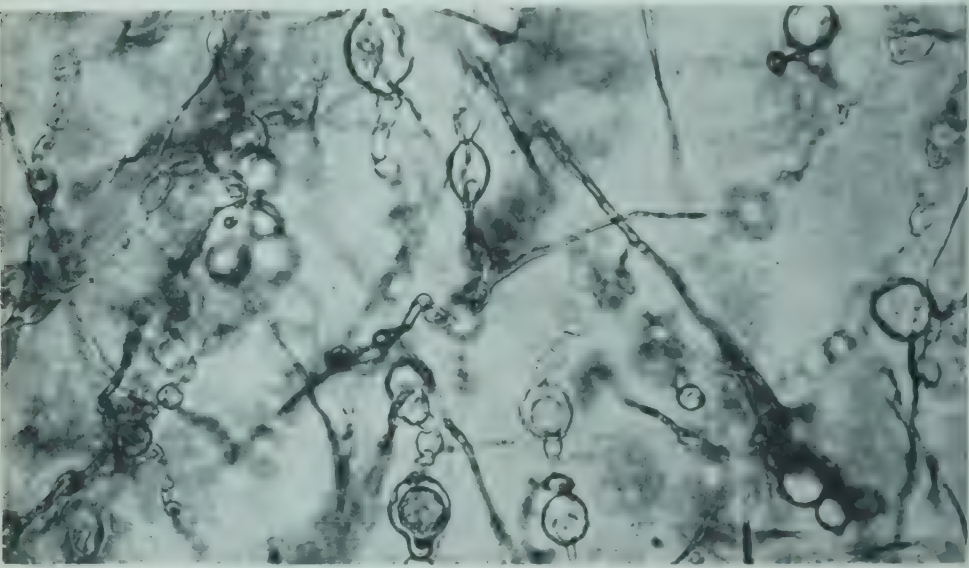

Fig. 7–9. Mold hyphae showing the development of chlamydospores.

Mold conidia may be produced singly or in chains which may be simple or branched. Usually a conidium is formed by the slight elongation of the terminal cell or tip of the conidiophore followed by the formation of a cell wall and by an abstriction of the spore. The tip then again elongates and pinches off another spore. It is evident that in this type the youngest spore is the one at the base of the chain. In a few cases the chain is formed by the repeated division or budding of the terminal cell of the chain. In this type the youngest spore is the one at the tip of the chain, i.e., the apical spore. Branching of the chain of spores may occur through the lateral budding of cells (as in *Hormodendrum,* see Fig. 7–22).

Sporangia are produced only by certain molds having a non-septate or coenocytic mycelium, though conidia are also produced by some of the members of this group. A sporangium originates as a terminal swelling of the sporangiophore or one of its branches; this enlargement is separated from the sporangiophore by the formation of a septum and enlarges considerably. The nuclei contained within it increase in number, and each surrounds itself by a bit of cytoplasm (Fig. 7–11). This is enveloped in a membrane, and spores are thus formed. The interior of the sporangium is in this manner more or less compactly filled with spores. Usually a structure known as the *columella* is also to be found within the sporangium.

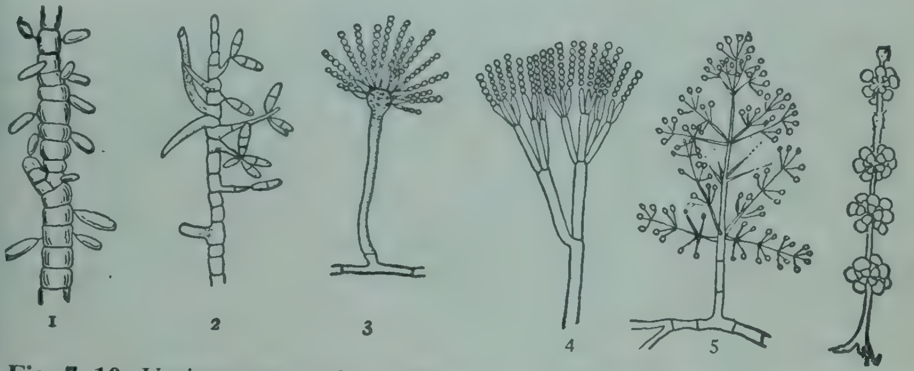

Fig. 7–10. Various types of conidiophores. 1. *Muelleriella,* a form in which conidiophores are absent, the conidia arising laterally directly from the mycelium. 2. *Urospora,* a form with simple conidiophores, little differentiated. 3. *Aspergillus,* with enlarged tip of well-differentiated conidiophore, and conidia arising in chains from the sterigmata. 4. *Penicillium,* with much-branched conidiophore. 5. *Acrostalagmus,* with conidiophores branched in whorls. 6. *Arthrobotrys,* simple conidiophore with spores borne in clusters from the enlarged nodes. (1, 2, and 4, adapted from Brefeld; 3, from Kny; 5 and 6 from Corda.)

Conidia are of varied shapes: spherical, oval, cylindric, fusiform (spindle-shaped), club-shaped, irregular, star-shaped, or greatly elongated and thread-shaped. They may be unicellular or multicellular (*septate*). The septa may be only cross partitions, or they may be longitudinal as well. A conidium divided into a number of cells by walls in different directions is said to be *muriform.*

Spores are usually much more resistant to unfavorable environment, such as heat, light, drying, and chemicals, than the molds that produce them. Many are well adapted for transportation by currents of air. When brought under favorable conditions for growth, they germinate and reproduce the vegetative mycelium.

Limitation of the Term Mold. The preceding discussion indicates that it is difficult, if not impossible, to formulate a wholly satisfactory definition for the term *mold*. It has been noted that the systematic botanist or mycologist does not recognize any such group of fungi. The only justification for the use of the term is an economic one, for the group contains many forms that are of importance in fermentation and in the preservation of foodstuffs and other organic materials. It is a group made up of fungi having certain superficial resemblances. All of them are alike in possessing a plant body made up of hyphae; most of them grow on many kinds of dead organic matter, frequently producing fermentation or decay; very few cause disease in man or animals. Many diseases of plants are also caused by closely related forms, but may be excluded from consideration here for they are of chief interest to the plant pathologist. In short, the group to be discussed is fairly well defined by the common conception of a mold as a more or less cottony, cobwebby, velvety, or powdery organism occurring on decaying or fermenting organic matter, and

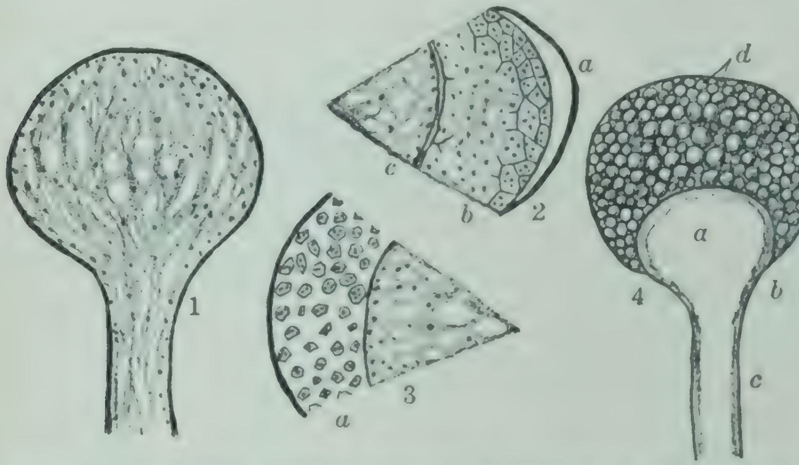

Fig. 7–11. Longitudinal sections through sporangia of *Rhizopus* at various stages of growth. 1. Sporangiophore tip swollen, protoplasm vacuolate, nuclei more numerous toward the periphery. 2. A wedge-shaped section from young sporangium at a little later stage than 1, the sporangial wall is differentiated at *a*, walls are cutting off groups of nuclei from each other to form spores at *b*, and the beginning of the wall of the columella is visible at *c*. 3. Similar to 2, but at a later stage in development, spores well separated from each other at *a*, columella wall distinct. 4. Mature sporangium, showing the columella (*a*), apophysis (*b*), sporangiophore (*c*), and spores (*d*) of various sizes. (Adapted from Blakeslee.)

not producing macroscopic fleshy fruiting bodies like the mushrooms and puffballs.

Botanical Relationships of the Molds. Organisms answering to the above general characterization of molds are found in at least three, probably in all of the four main subdivisions of the fungi, namely, the *Phycomycetes, Ascomycetes,* and *Fungi Imperfecti* (possibly the fourth, the *Basidiomycetes,* should be included also). A brief discussion of these groups follows.

Phycomycetes. These fungi are distinguished by possession of a non-septate or little septate mycelium and by producing sexual spores directly as the result of the union of two gametes. Usually asexual spores are produced as well, frequently in sporangia, more rarely as conidia. Of the families belonging to the *Phycomycetes,* one only, the *Mucoraceae,* contains organisms usually included with the molds. The commonest of these is *Rhizopus,* the black mold on bread.

Ascomycetes. The *Ascomycetes* are differentiated by the development of the cell resulting from fertilization into a group or mass of cells, some of which become spore sacs or asci, each containing typically four or eight spores. Usually asexual spores (conidia) are produced as well. In many species the formation of asci and ascospores is of exceptional occurrence, multiplication usually being by means of the conidia. As will be noted later, probably many forms have altogether lost the power of ascospore production and reproduce asexually exclusively. Several of the commonest of the mold genera belong to the *Ascomycetes,* such as the green and blue-green molds of oranges, apples, and bread.

Basidiomycetes. All produce typical spore-bearing structures called basidia. Few of these forms would ever be mistaken for molds; they are for the most part the rusts, smuts, puffballs, mushrooms, and toadstools. None of them will be discussed here.

Fungi Imperfecti. All fungi that do not fall into one of the three preceding groups are placed among the *Fungi Imperfecti.* Probably in most cases they are *Ascomycetes* whose perfect or ascus stages have never been found or that have wholly lost the ability to produce ascospores. Most of the molds to be considered belong in this group.

CLASSIFICATION OF THE MOLDS

The molds are sometimes grouped together for convenience with the name *Hyphomycetes,* ranking with the classes noted above. It is evident that this is not in strict accordance with relationships and does not represent an evolutionary classification. It possesses the advantage, however, of facilitating the construction of a key which renders identification of the various common mold genera comparatively simple.

Several hundred genera and thousands of species of *Hyphomycetes* have been described, scattered among five families. Of these genera, however, a relatively small number includes most of the molds commonly met in the home, in the laboratory, and in industry. The criteria used in telling the different kinds apart are the various morphological characters which have previously been enumerated. A key which will permit the identification of the genus of most of the commoner molds is included in the Appendix. The following key will facilitate the determination of the genus of most of the mold species encountered in the laboratory. This key is based wholly upon differences in production of asexual spores. It is followed by more detailed descriptions of the genera, and in some cases of certain important species. These descriptions of the genera should be consulted before assuming the accuracy of a determination made by this key. If the mold under study does not agree, use the key in the Appendix.

Key to the Genera of the Most Common Molds

a. Spores (sporangiospores) borne in sporangia. (Order ***Mucorales***)
  b. Sporangiophores arising from holdfasts or rhizoids at the nodes of the stolons. *Rhizopus*
  bb. Sporangiophores arising singly, no rhizoids or stolons. *Mucor*
aa. Spores (conidia) never borne in sporangia. (Order ***Moniliales***)
  b. Neither hyphae nor conidia smoky or dark in color.
    c. Conidia one-celled.
      d. Conidia (arthrospores) formed by segmentation of hyphae. *Oöspora* (*Geotrichum*)
      dd. Conidia (not arthrospores) not formed by segmentation of hyphae.
        e. Conidiophores unbranched, enlarged at apex. *Aspergillus*
        ee. Conidiophores branched.
          f. Forming a brushlike mass, spores in unbranched chains. *Penicillium*
          ff. Not forming a brush; spores in branched chains. *Neurospora* (*Monilia*)
    cc. Conidia two-celled, pear-shaped. *Trichothecium*
  bb. Either hyphae or conidia dark or smoky, frequently both.
    c. Conidia one- or two-celled. Chains of conidia may branch
      d. Conidia one-celled. *Hormodendrum*
      dd. Conidia becoming two-celled when old. *Cladosporium*
    cc. Conidia many-celled, muriform, in chains. *Alternaria*

Order Mucorales. The mycologist includes this as one of the orders of the algal-fungi or *Phycomycetes*. It is characterized by the formation of *zygospores*. These zygospores are not commonly produced by many species, hence the asexual spores and their arrangement furnish a somewhat simpler and more useful means of classification. Most of the genera produce sporangia; a few do not, asexual reproduction then being by means of conidia. The two most important genera are the black molds *Rhizopus* and *Mucor*.

Rhizopus. Species belonging to this genus are commonly found on moldy vegetables, grain, and bread. The spores are usually present in the laboratory air, and these molds are common contaminants of cultures. The vegetative mycelium penetrates the material or substrate on which the mold is growing and soon sends up into the air long, slender threads known as aerial hyphae or stolons. These grow until they come in contact with some solid object such as the wall of the vessel; they then produce clusters of rootlike holdfasts or *rhizoids* (Fig. 7–12). These are plainly visible through the glass sides of a flask or a petri dish in which the mold is growing. The stolon may continue its growth and again throw out rhizoids. From each of the points where the rhizoids are produced, there develops a cluster of unbranched sporangiophores. The sporangium which develops at the tip of each contains a large number of spores. When young it is white, but soon turns brown or black, due to the ripening and darkening of the spores. A columella is present but is united through part of its length with the sporangium wall. The sporangium wall breaks to pieces readily when mature and releases the spores.

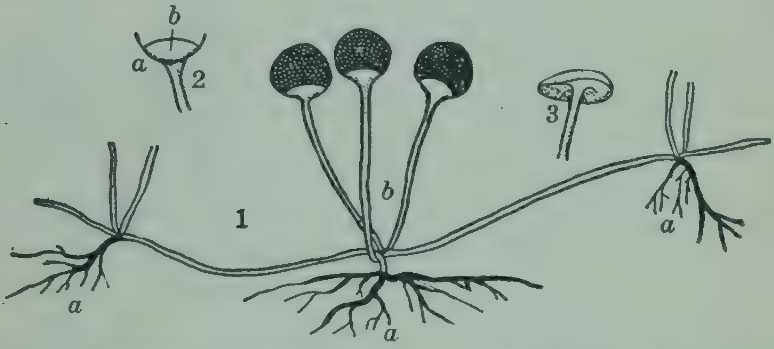

Fig. 7–12. *Rhizopus nigricans*. 1. Growth habit of the mold, showing the stolons, the rhizoids (*a*), cluster of sporangiophores (*b*), with sporangia filled with spores. 2. Lower portion of a sporangium to show the apophysis (*a*), and columella (*b*). 3. Lower portion of sporangium with a collapsed columella. When mature sporangia are examined microscopically, particularly when mounted in water, the columella collapses to form an umbrella.

The columella frequently collapses when mounted in water for microscopic examination, the lower half of the hemisphere invaginates, and the whole assumes the appearance of an open umbrella with the sporangiophore as the handle (Fig. 7–12).

To the naked eye *Rhizopus* is a somewhat coarse, fuzzy, or cottony mold, frequently growing over a considerable area of the substrate. When the sporangia have formed, it becomes grayish due to the presence of great numbers of black spheres just visible to the eye.

The most common species of this genus is *Rhizopus nigricans*, the black bread mold. It is found on all sorts of decaying vegetables, fruits, and meals. *Rhizopus oryzae*, *R. japonicus*, and several other species have been described from fermenting meals and grains. Some will be discussed later as of importance in the manufacture of industrial alcohol.

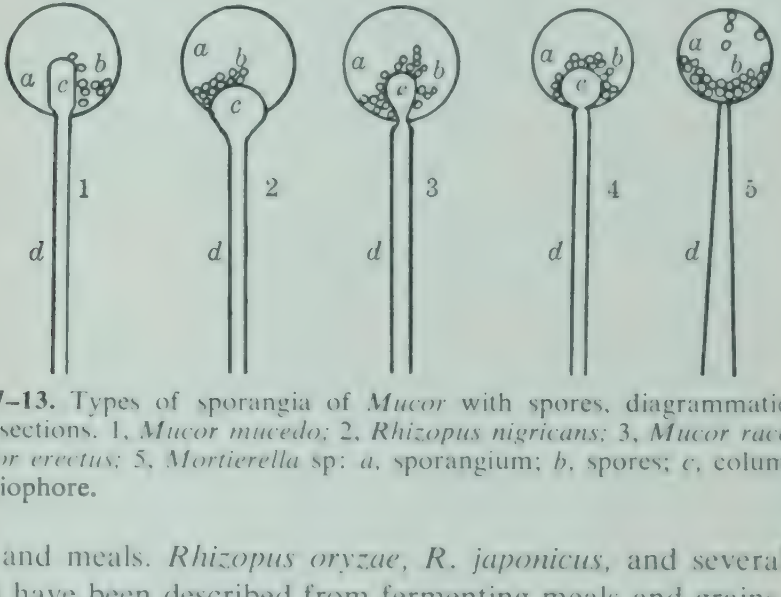

Fig. 7–13. Types of sporangia of *Mucor* with spores, diagrammatic longitudinal sections. 1, *Mucor mucedo*; 2, *Rhizopus nigricans*; 3, *Mucor racemosus*; 4, *Mucor erectus*; 5, *Mortierella* sp: *a*, sporangium; *b*, spores; *c*, columella; *d*, sporangiophore.

Mucor. Mucors are common on decaying vegetables, fruit, bread, etc. This genus differs from *Rhizopus* principally in that there are no stolons. The sporangiophores do not arise in clusters but are produced singly. In some species they branch. The sporangium closely resembles that of the *Rhizopus*, the columella is usually not as large, and the sporangium wall is frequently somewhat more resistant. To the naked eye a *Mucor* (like *Rhizopus*) is a somewhat coarse, cottony mold. The different species are distinguished from each other largely by branched or unbranched sporangiophores, shape of columella, and size and shape of sporangium and spores (Figs. 7–14 and 7–15). Some of the species of *Mucor* produce heavy-walled resting cells or *chlamydospores* in the vegetative mycelium (*Mucor racemosus*). One *Mucor* (*M. rouxii*) has been used in the saccharification of starch for the purpose of alcohol production.

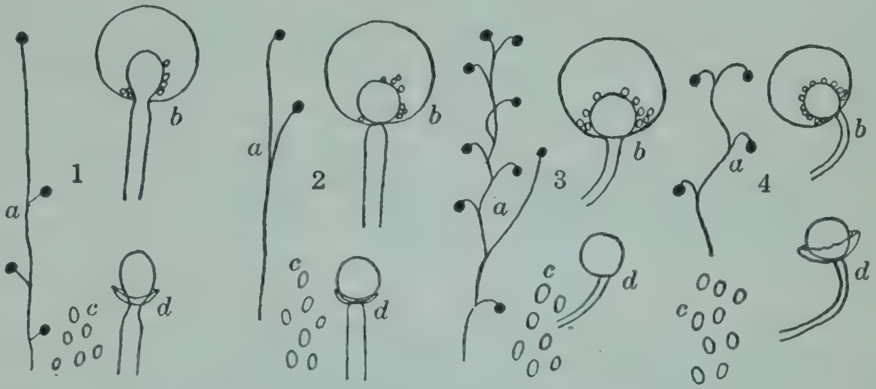

Fig. 7–14. The principal species of *Mucor: a,* sporangiophore and sporangia; *b,* longitudinal section through a sporangium; *c,* spores; *d,* columella with the sporangial wall fallen away. 1, *Mucor racemosus;* 2, *M. erectus;* 3, *M. circinelloides;* 4; *M. alternans.* (1, adapted from Riess; 2, from Bainier; 3, from Gayon and Subourg; 4, from Gayon.)

Order Moniliales. This order is one of the subdivisions of the *Fungi Imperfecti,* but as here used includes some genera in which some species may produce asci and ascospores, and definitely belong to the *Ascomycetes,* in other species asci have never been found, and identifications must be made almost entirely upon the characteristics of the asexual spores or conidia. The spores or conidia are never borne in sporangia.

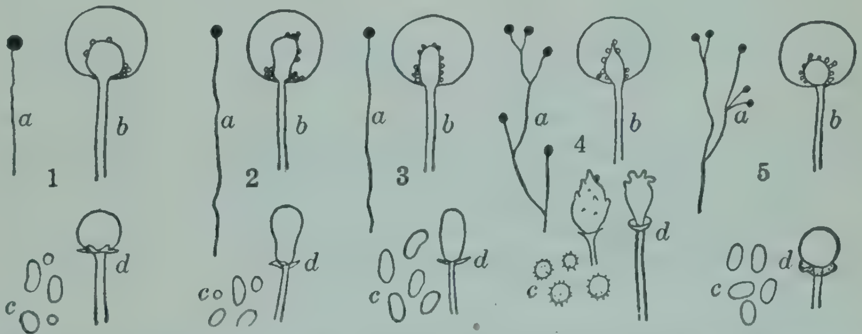

Fig. 7–15. The principal species of Mucor continued. *a,* sporangiophore and sporangium (or sporangia); *b,* single sporangium in longitudinal section; *c,* spores; *d,* columella with sporangium wall fallen away. 1, *Mucor hiemalis;* 2, *M. piriformis;* 3, *M. mucedo;* 4, *M. plumbeus;* 5, *M. rouxii.* (1, 2, 3, and 5 adapted from Wehmer; 4, from Gayon.)

Oöspora (*Geotrichum*). One species is of considerable importance, the *Oöspora lactis*[2] found commonly on soured milk, in cheese, and in other milk products. The mycelium of this fungus grows almost entirely within the nutrient substratum, but chains of spores formed by the segmentation of aerial hyphae may project above the surface. The vegetative mycelium itself also frequently segments into conidia (arthrospores). This type of spore formation within the substratum is quite characteristic. The dichotomous branching of the mycelial threads is also somewhat unusual; they often fork into two equal branches, instead of one continuing as a main thread and the other as a lateral offshoot.

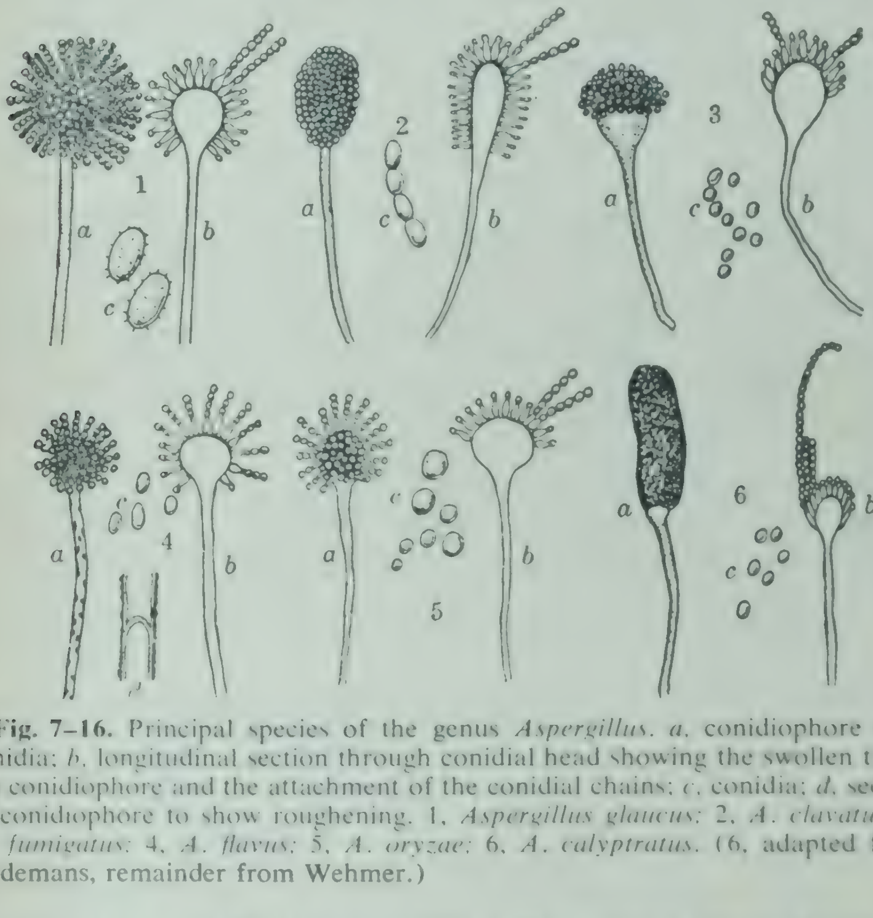

Fig. 7–16. Principal species of the genus *Aspergillus*. *a*, conidiophore with conidia; *b*, longitudinal section through conidial head showing the swollen tip of the conidiophore and the attachment of the conidial chains; *c*, conidia; *d*, section of conidiophore to show roughening. 1, *Aspergillus glaucus*; 2, *A. clavatus*; 3, *A. fumigatus*; 4, *A. flavus*; 5, *A. oryzae*; 6, *A. calyptratus*. (6, adapted from Oudemans, remainder from Wehmer.)

Aspergillus. Representatives of this genus are common upon decaying vegetables, moldy corn, and grain, as greenish, yellow, orange, brown, or black, velvety or powdery molds. The spores are common in the air and may develop in imperfectly sterilized laboratory media or in media that have been exposed to dust contamination. This genus belongs among the *Ascomycetes,* for many species produce ascospores. The asci and ascospores are borne in spherical golden yellow *cleistothecia,* usually lying closely appressed to the vegetative mycelium. The characteristic method of bearing conidia renders recognition of this genus relatively easy. The conidiophores are unbranched and usually long. Each develops from a special enlarged cell of the mycelium termed a *foot* or a *branch cell.* It is swollen or enlarged at the tip into a *vesicle,* upon the surface of which there develop numerous short stalks, usually set close together, giving to the end conidiophore the appearance of a war club with spikes. These small stalks are termed by some authors *phialides* (sing., *phialis*); by other authors *sterigmata* (sing., *sterigma*). They may be simple or branched; if the latter, the basal branch is termed a *metula.* From the tips of these phialides spores are abjointed, forming chains. These chains under favorable conditions may be relatively long. Many species of *Aspergillus* have been described. They may be separated into groups on the basis of the color of the mature spore masses. A key to some of the commoner species is given in the Appendix.

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[2] Also known as *Geotrichum candidum*. There is some question as to whether *Oöspora* or *Geotrichum* is the correct generic designation. The generic name *Oidium* is also sometimes (incorrectly) used.

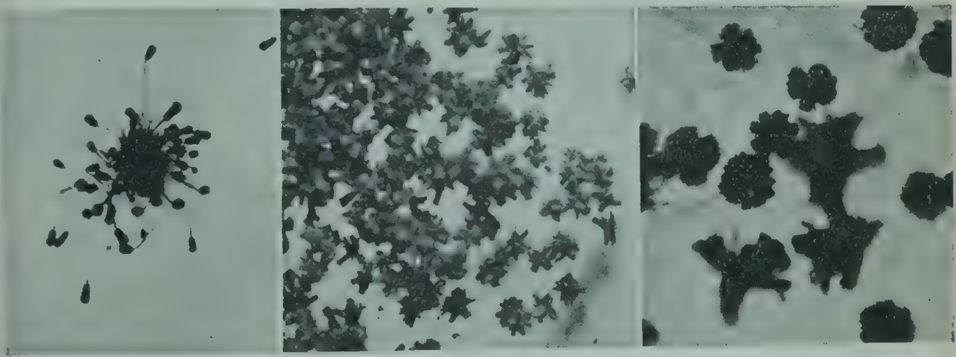

Fig. 7–17. Aspergillus. A. Low-power photograph of a small colony of *Aspergillus* showing the conidiophores and conidial heads. In this species the chains of conidia tend to remain closely appressed. B and C. Heads showing the radiating chains and clusters of chains of conidia.

Of the black spored forms, *Aspergillus niger* is of importance because of its active fermentative ability in sugar solutions. It is used for the commercial production of citric acid; under suitable conditions oxalic acid may be formed. This and other species bring about many other chemical changes to be noted later. It has been much used in the laboratory studies of mold nutrition, metabolism, and enzyme action. *A. glaucus* is one of the commonest of the green-spored species on moldy grain, silage, canned fruits, and jellies. It usually produces golden yellow perithecia in abundance in the older cultures. *A. fumigatus* produces a fatal pneumonia in birds when inhaled and is believed to be pathogenic for animals. The commonest of the white or cream-spored forms is *A. candidus,* of the yellowish green *A. flavus,* and of the yellow *A. ochraceus. Aspergillus oryzae* has been used commercially in the conversion of starch to sugar preliminary to its fermentation by yeast for the production of alcohol.

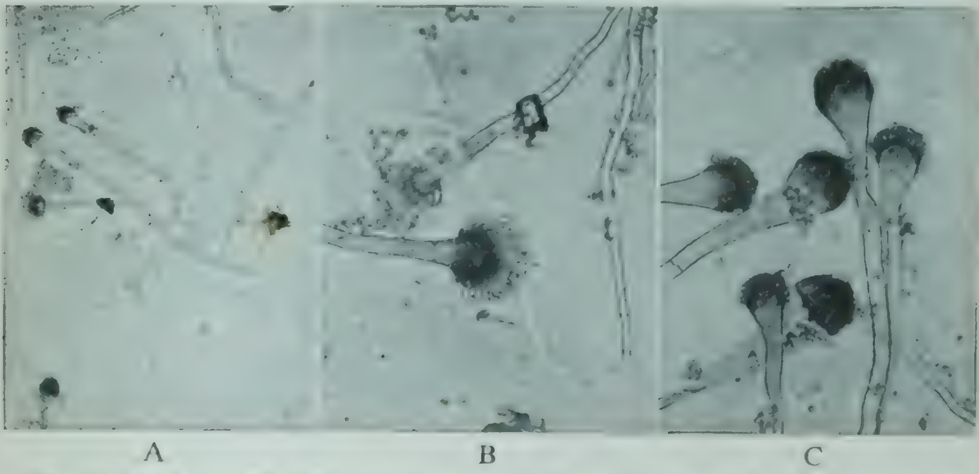

Fig. 7–18. *Aspergillus.* Photomicrographs of conidiophores, heads, sterigmata. (phialides) and conidia. A. Note that the cell of the hypha from which the conidiophore branches is heavier walled; it is the *basal cell.* In conidiophore to right the other hyphae to left and right are scarcely visible. B. Heads with branched sterigmata. C. Heads with single row of sterigmata.

Penicillium. This genus is closely related to the preceding and is classed among the *Ascomycetes* although the formation of asci and ascospores is very rarely observed and possibly never occurs with many species. The representatives of this genus are probably the most common of all molds. They are the blue-green molds observed on oranges, lemons, apples, and other fruits, vegetables, preserves, grains, hay, in short on almost any moist organic substance. The genus is differentiated from *Aspergillus* by the manner in which the spores are borne. The vegetative mycelium penetrates the substratum, and later sends up erect aerial hyphae as conidiophores. These branch one or more times in whorls, giving rise to a terminal cluster of parallel

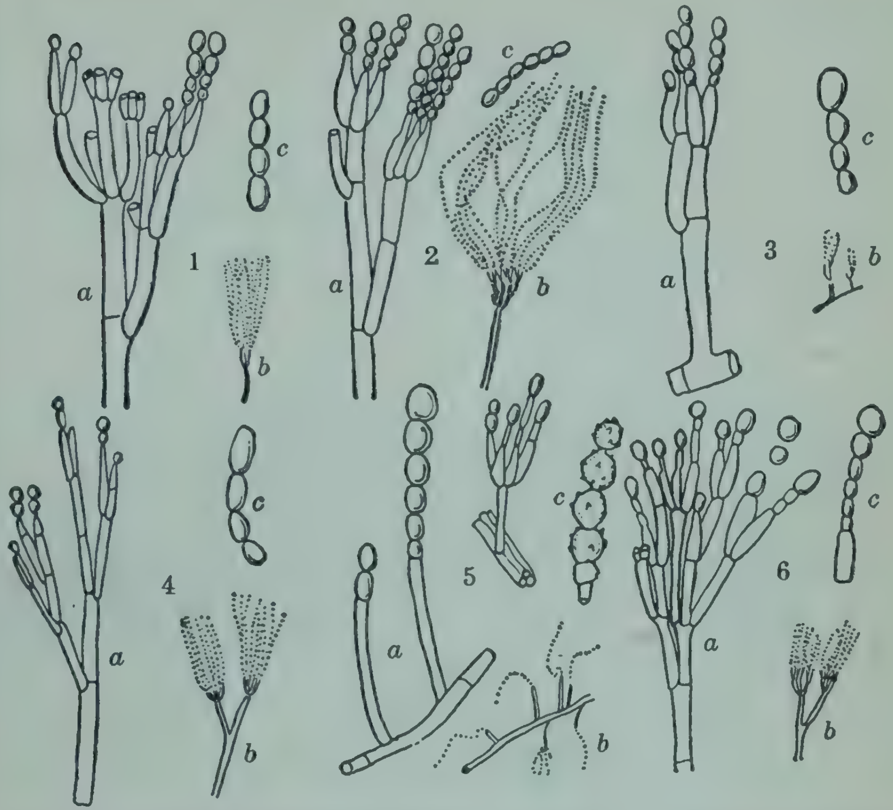

Fig. 7–19. Common species of the genus *Penicillium*. *a,* characteristic branching of the conidiophores; *b,* same on a smaller scale; *c,* conidia. 1, *Penicillium expansum;* 2, *P. italicum;* 3, *P. digitatum;* 4, *P. roquefortii;* 5, *P. brevicaule;* 6, *P. camembertii.* (Adapted from Thom.)

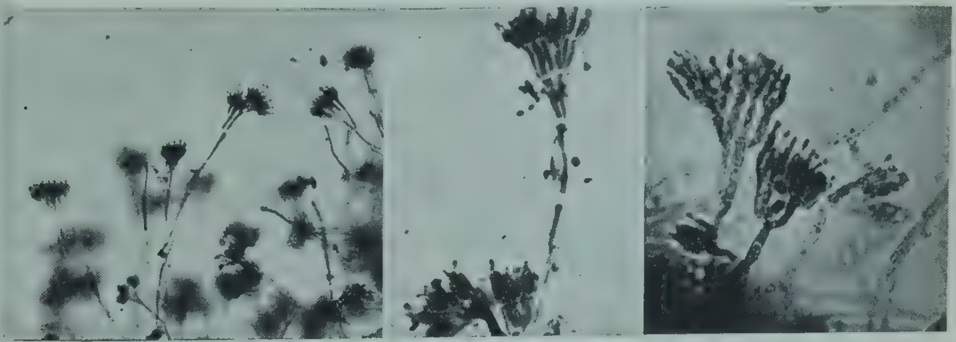

Fig. 7–20. Conidiophores of *Penicillium* showing the branching at the tip, the conidia are produced in chains. The conidia have fallen from the tips as a result of manipulation in preparation.

hyphae; of these the ultimate branchlets are to be regarded as phialides (sterigmata). From each of them conidia are abstricted in a chain. The branches and conidia together resemble a broom or a camel's-hair brush, hence the name *Penicillium*[3] (Fig. 7–21).

As in *Aspergillus*, the color of the mature mold is used as a basis for separation of groups of species, but is not altogether reliable as it may vary somewhat with environment. Several species have been shown to be of importance in the ripening of certain types of cheese, as *Penicillium roquefortii* in Roquefort cheese and *P. camembertii* in Camembert cheese. *P. expansum* is common on many decaying substances, particularly on apples. When growing under favorable conditions, considerable numbers of the conidiophores may unite into a white stalk bearing the green spores at the tip. One of these compound conidiophores readily visible to the unaided eye is termed a *coremium*. *P. glaucum* is the name that was first given to a green *Penicillium* and is frequently used to indicate any one of this group. Altogether several hundred species of Penicillium have been described. The differentiation of the species of *Penicillium* is relatively difficult, so no keys are included. *Penicillium notatum* will be discussed in some detail in consideration of the production of antibiotics, particularly penicillin.

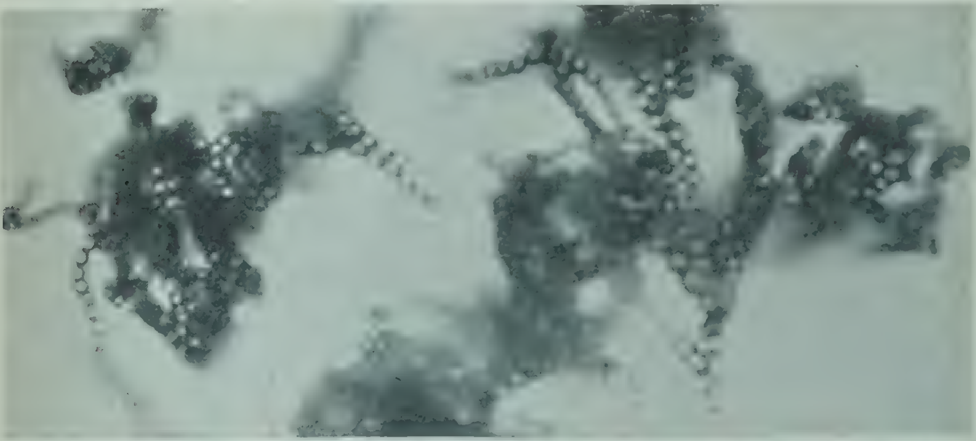

Fig. 7–21. *Penicillium.* Chains of spores or conidia arising from the tips of the fertile branches.

Neurospora (*Monilia*). Molds of this genus are not uncommon on moldy bread, grain, and decaying vegetation. The conidia are brightly colored in some species, pink, red, or orange. The conidia are produced in large numbers from the tips of branched conidiophores, not arranged in the form of a brush as in *Penicillium*. The chains of conidia also branch. In general the perfect or ascus stage is not produced by a strain originating from a single spore; the mycelia are of at least two types, and it is only when cultures of the two types come in contact that the sexual stage can be achieved and asci and ascospores produced. Fungi of this type are said to be *heterothallic*. The asci of *Neurospora* and the (usually) eight spores contained are relatively large. The spores can be readily isolated and the mold grown in pure culture.

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[3] From the Latin *penicillus,* a little brush.

The genus *Neurospora* has come to be one of the most studied groups of fungi. It is important in certain foods, in some cases as a cause of spoilage, in others as bringing about desirable fermentations. It has been found to be peculiarly significant in the study of inheritance, as will be discussed in Chapter 8.

In part as a result of inheritance studies it has been found particularly useful as a tool in the study of the steps in certain chemical changes which are produced in living cells. Some of these will be noted later. Two species have been studied most extensively, *Neurospora sitophila* and *N. crassa.*

Trichothecium (*Cephalothecium*). The members of this genus are not uncommon on decaying fruits, particularly apples. The mycelium penetrates the substratum and sends into the air at intervals straight unbranched conidiophores. The spores may be solitary or in a loose cluster. The conidia are two-celled, one cell frequently being larger than the other. The most common species is *Trichothecium roseum*. The specific name comes from the faint rose or pink color of the mold, particularly of the spores. This mold is common on the apple and sometimes causes considerable damage through the development of a rot. The spores are not uncommon in the air and develop readily on culture media in the laboratory.

Hormodendrum and Cladosporium (Fig. 7–22). These genera are so closely related and so intergrade into each other that they will be considered together. They are common on decaying paper, wood, fruits, and vegetables, usually producing dark or sooty patches. They are to be recognized by the dark or smoky appearance of the mycelium and usually of the spores as well. The latter are borne in more or less irregular branching chains. The conidia of *Hormodendrum* are unicellular. *Cladosporium* produces two-celled spores, at least in old cultures, but in young cultures the mold closely resembles *Hormoden-*

drum. Cladosporium herbarum is one of the commonest of molds encountered in the laboratory. In each of these forms the vegetative mycelium of brown, much-branched hyphae penetrates the substratum and sends up more or less well-differentiated conidiophores above the surface.

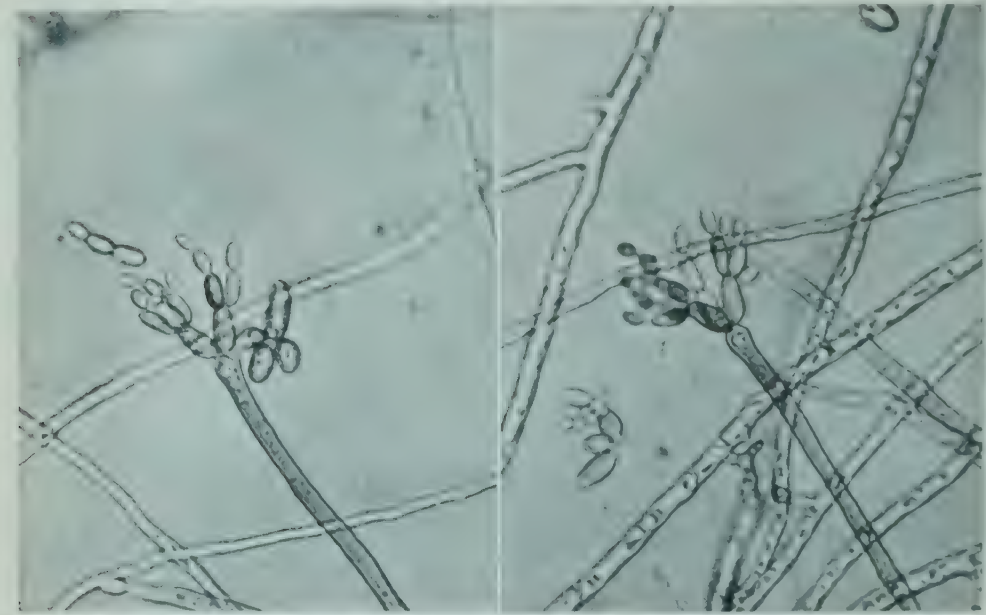

Fig. 7–22. *Hormodendrum.* Conidiophores and conidia. Note that the conidia and fertile hyphae (conidiophores) are dark in color, and that the conidia bud from the tip of the conidiophore and from other conidia. The youngest conidium is at the tip of the chain.

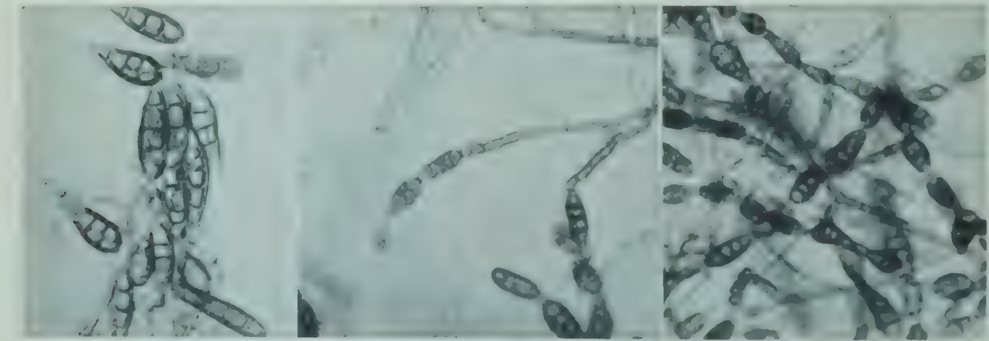

Fig. 7–23. *Alternaria.* Photomicrographs showing the muriform spores or conidia, the colorless vegetative mycelium, the dark conidiophores.

Alternaria. The molds belonging to this genus have large, brown, many-celled spores borne in chains on short conidiophores (Fig. 7–23). The septa in the spore occur both at right angles and parallel to the long axis, and the spore is said to be *muriform*. Many species of *Alternaria* have been described from decaying vegetation. The spores are common in the air. The most abundant species is probably *Alternaria tenuis,* found on moldy grain, seeds, leaves, hay, in the soil, and commonly infecting laboratory media. Some species are believed to cause disease in certain plants.

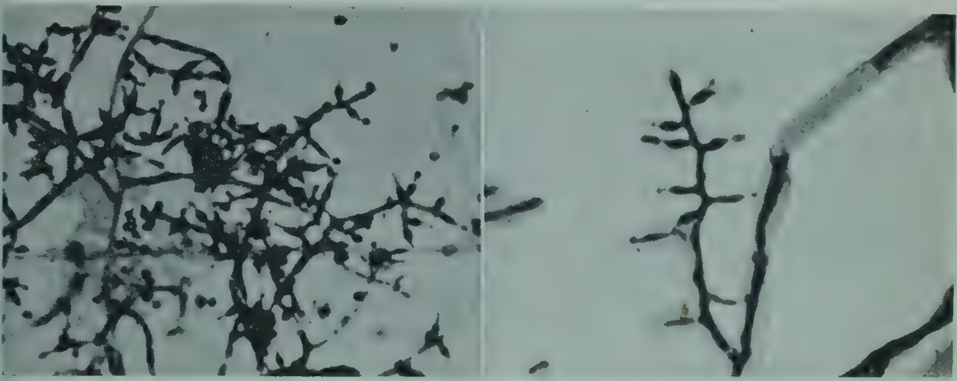

Fig. 7–24. *Trichoderma.* Photomicrographs showing the vegetative hyphae, the branched conidiophores, and the development of the conidia.

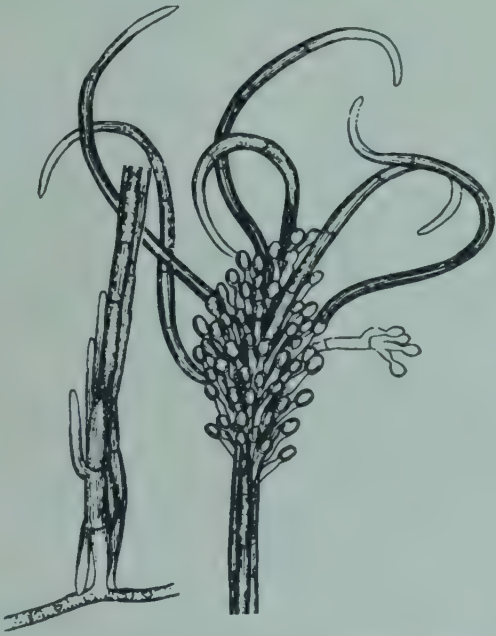

Fig. 7–25. *Trichurus.* Conidiophores, sterigmata, conidia, and sterile threads. (Adapted from Hasselbring.)

CHAPTER 8

Inheritance, Variability and Mutations of Microorganisms

Do microorganisms resemble higher plants and animals in the way in which they pass on their characteristics to their progeny? Are the same kinds of mechanisms at work in these cells as are evident in other groups of living things? And are the explanations of the nature of variations the same?

The minute size of the bacterial cell has led to much speculation as to the presence of a nucleus. In fact, some students in the past have insisted that one of the truly distinctive characteristics of the bacterial cell is the absence of a nucleus. As was pointed out in the discussion of the morphology of the bacteria, the evidence brought forward by the use of the newer techniques seems clearly to indicate that the internal organization of the bacterial cell is not unlike that of the fungi. The yeasts and molds quite definitely have nuclei with chromosomes which function very like those of higher forms. There is evidence that even the minute rickettsias show some internal differentiation and organization.

The viruses pose quite another problem. The size of the entire virus particle is about that calculated for a gene; perhaps it may be regarded as subcellular and quite without a nucleus. Yet, as will be shown, viruses may show a definite inheritance pattern.

Microorganisms, like all other forms of life, are quite variable. In comparatively recent years experiments based upon good genetic techniques have begun to bring about some understanding of the classification and origins of these variations. Many major contributions have been made to genetics, as the discipline which studies heredity and variation and their interrelationships, through critical observation of many microorganisms. Some of them have proved as adaptable and significant in plant genetics as have the fruit fly (*Drosophila*) and the mouse in animal genetics.

The earlier basic studies of heredity were made for the most part upon animals and plants that reproduce sexually. The development of the cellular concept of organization was followed by studies of the cell nucleus and particularly of the chromosomes. The essence of sexuality was determined to be the union of the nuclei of the sex cells without loss of the individuality of the chromosomes. The nucleus of the cell resulting from fusion of sex cells carries one of each chromosome from each of the parents; the corresponding chromosomes are termed homologous. The plant and animal bodies of higher forms generally are composed of cells which contain the doubled or *diploid* number of chromosomes. In ordinary cell division each chromosome splits lengthwise, one-half of each homologous chromosome goes to each pole. However, in the divisions of the cells leading immediately to the formation of sex cells there is a series of cell divisions (*meiosis*) in which one of each of the homologous chromosomes goes to one pole, and one to the other. The sex cells thus formed contain nuclei each with one only of each of the homologous chromosomes, and the cell is said to be single or *haploid* as to number of chromosomes.

Then came the discovery that expression of certain characteristics of the animal or plant seemed to be linked to certain chromosomes and that each chromosome contained many to few elements which apparently governed the inheritance of particular characteristics. These heredity units or components of the chromosome (called *genes*) were shown to be ranged linearly along the chromosome. In the formation of the germ or sex cells the homologous chromosomes are randomly distributed to the daughter nuclei; this means that each sex cell may have one or more chromosomes derived from each parent. This random distribution leads to a variety of kinds of genes in a sex cell (*gamete*), and still greater variety in the diploid cell originating from the fusion of these differently constituted sex cells. Remarkable advances have been made in linking definite morphological characters in the diploid plant or animal with genes located at certain positions on definite chromosomes. The number of genes apparently is very large, so workers studying particular plants as corn, or animals as the fruit fly, gradually have developed genic maps of the several chromosomes.

What evidence, if any, is there that microorganisms follow the pattern of the higher forms? Is there evidence of the existence of genes? Are there any sexual methods of reproduction that are comparable to those of the plants and animals on which most of the work has been done?

Particularly are we concerned with such questions as evidences of similarities and differences in the mechanism of inheritance in the microorganisms as contrasted with inheritance in higher plants and animals. Even though sexual reproduction has not been *proved* to exist in all microorganisms, it seems probable that certain of the findings of genetics in higher forms may be extended to the microorganisms and proved valid. As stated by Pontecorro (1948):

> The techniques and concepts of classical genetics are valid and applicable wherever there is a factorial basis for heredity and variation, no matter whether the factors are, like the genes, mechanically organized to form chromosomes or, like the viruses, free particles inside the cell.

Progress in solution of many problems in genetics has been greatly accelerated by use of animals and plants that have relatively short developmental cycles. The fruit fly (*Drosophila*) which can complete a life cycle from egg to mature fly to egg in a relatively few days has given understanding which would require years and decades had it been necessary to use higher animals with long life cycles. Similarly, studies on plants have been greatly expedited in recent years by use of certain fungi, notably certain of the moldlike sac fungi (*Ascomycetes*) such as *Neurospora*. This organism has proved to be so significant in research and the facts discovered by its study have such an important bearing on microbiological concepts as to warrant a brief description.

The genus *Neurospora* includes several molds which are relatively common. They may be readily cultivated in the laboratory. Study has shown that cells of the hyphae of a mold colony that has developed from a single spore contain nuclei with the haploid or reduced number of chromosomes. Further, it has been found that the sexual cycle is never initiated by a single plant. About half of the plants are of one sex, and half of the other. Inasmuch as there seems to be no recognizable morphological difference between the two kinds of plants, it is difficult to call one male and one female; instead one type may be called the plus (+) strain and the other the minus (−) strain.

When the hyphae of two plants, one + and the other —, grow together, there is a fusion of cells from the respective plants in pairs, a primitive sexual process. The resultant cell nucleus now contains the doubled number of chromosomes and is diploid. The cell grows and multiplies to form a mass of cells which together make up a fruiting body called a perithecium, within which there are produced some large elongate cells, each with its diploid nucleus. This nucleus then divides, then the two daughter and the four granddaughter nuclei divide, with resultant eight nuclei. Each nucleus is surrounded by a layer of cytoplasm, and by a cell wall, constituting an ascospore, the eight ascospores being arranged in a row in the ascus formed from the mother cell. Study of the spores produced in each ascus reveals that in the process of spore formation there has been a reduction from the diploid to the haploid condition. It is possible by means of careful micro-manipulation to dissect out a single acus and to remove and germinate each of the ascospores separately. It will be found that usually four of the fungus colonies produced are + and four are —.

The mold colonies of *Neurospora* also produce asexual spores or conidia which when placed under favorable conditions will produce a mold colony of the same characteristics and type as the parent colony.

Observation shows that occasionally the mold colony may produce a hypha differing in some character such as color, morphology, physiologic behavior, or nutritional requirements. And the conidia produced from such a mutant hyphal branch will reproduce the modified type of mold. Inasmuch as definite genes are believed to produce the characteristic modification, the phenomenon is interpreted as the result of a modification of a gene, and the consequent change in color, morphology, or physiology as due to the change in the corresponding gene. Such a change is inherited, and the resultant organism is called a *mutant*. It has been found that the number of these mutations can be very greatly increased by exposing the mold colonies to radiation of certain types, such as x rays or to ultraviolet light. Many kinds of mutations have been thus induced.

What happens when a mutant strain is mated with another strain differing in some one characteristic in addition to sex? Let a strain that is + and produces red conidia be mated with one which is — and has colorless conidia. The fusion of the gametes gives rise to diploid cells from which the asci and ascospores developed, and the eight spores (haploid) can each be isolated, germinated, and grown.

What kinds of colonies will be produced? Usually, it will be found that four kinds of colonies will develop, two will be red +, two red —, two colorless +, and two colorless —.

Of special interest is the fact that these mutations may be physiological. *Neurospora* of the "wild" type will grow on a medium of relatively simple composition, including a source of carbon such as sugar, a nitrogen source such as an ammonium salt, certain inorganic salts, the vitamin biotin, and water. It is able from these to manufacture all the varied organic compounds which are needed for the growth of the fungus. Such manufactured compounds are very numerous, for it is known that the chemical changes brought about by the large number of enzymes produced by the cell are likewise numerous. Among the mutants resulting from radiation or other treatment will be found some which have completely lost their ability to produce some one substance essential to growth. If this single compound is added to the medium, the organism is able to grow, but cannot in its absence. For example, a mutant may be developed which requires for its growth the addition of methionine, a compound which the normal plant can make for itself. Particularly noteworthy is the fact that studies on the inheritance of this characteristic seem to indicate that this lack of ability to produce methionine is determined by the mutation of a single gene. In other words, the ability of the cell to produce some enzyme needed in the synthesis of the methionine is linked to a particular gene; alteration of this gene (mutation) deprives the cell of the required enzyme.

The results of studies of the type just outlined have been far-reaching; they have contributed a great amount of information relative to fundamental genetics. It has been possible to demonstrate that many substances synthesized by plants and necessary for their growth result from a series, sometimes a rather long series, of chemical reactions, each one catalyzed by a different enzyme. A block at any step in this chain of reactions is sufficient to stop the development of the end product. Further, mutants are known in which the block is at different points in the chain. If the series may be represented as,

$$A \xrightarrow{1} B \xrightarrow{2} C \xrightarrow{3} D \xrightarrow{4} E$$

the arrows represent changes catalyzed by enzymes 1, 2, 3, and 4. Production of the required E can be prevented if any one of the enzymes is lacking. Obviously, if enzyme 2 is lacking, A can be changed to B, but C, D, and E cannot be formed. However, if C or D is supplied to the organism, obviously E can be produced and growth proceed. But addition of B will not permit synthesis of E. Not only has study with different compounds and gene mutants been helpful in establishing the sequence of changes in elaboration of a new end product, but it has also yielded to the biochemist most valuable tools for the qualitative and even quantitative analyses for definite compounds. As an example, the normal "wild" strains of *Neurospora* can synthesize the required nicotinamide. The sequence of synthesis has been found to be:

anthranilic acid → indole → tryptophan → kynurenine → nicotinamide

Similar studies have been made with other molds and fungi that show a definite sexual phase. Of special interest was the discovery that similar results could be secured with the true yeasts, that some possess both + and — strains whose cells will conjugate and produce asci and ascospores. It has proved possible to produce mutants in yeast by irradiation or by use of certain chemicals and to cross these mutants,. securing strains of yeast with new or previously unknown combinations of characters.

Mutants of bacteria are also well known. Spontaneous mutation is not uncommon in bacteria, and the mutants can be isolated by use of appropriate techniques. For example, certain species of bacteria are known which can produce acid from the sugar glucose in a suitable medium, but not from lactose. However, if the organism is placed in a culture medium otherwise capable of permitting growth, and containing lactose as the only sugar, after a period of time the medium will be found to be acid. Does this mean the gradual modification of all the growing cells so that lactose can be attacked, or alternatively does it mean that an occasional bacterial cell mutates with development of lactose-fermenting power? Careful study has shown the latter to occur in this case.[1] Such mutations in physiological characters occur infrequently, perhaps not oftener than once in the course of a million or even ten million cell divisions. But in a culture of bacteria the cells soon are numbered in the millions. If a single mutation of a cell occurs with development of ability to produce an enzyme which will enable it to ferment lactose, that cell has an advantage over other cells present in that it can utilize a nutrient not available to the others. By appropriate methods it is possible to show that the mutant (lactose-using) form grows and multiplies more rapidly and soon becomes dominant in the culture. From such a culture one may secure two kinds of cells, some like the parent incapable of fermenting lactose, the other the mutant which in culture continues able to ferment lactose. This mutant when grown in the absence of lactose does not lose the lactose-fermenting ability.

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[1] Not all development of ability to produce new types of chemical change are due to mutations; see later discussion of constitutive and adaptive enzymes.

Such mutations are known to occur not infrequently in the bacteria, but unless the mutation is one which proves advantageous to the mutant, it is usually "smothered" in the great mass of other cells.

Bacteria, like the yeasts and molds, produce mutations in much greater numbers when exposed to radiation (such as x rays) or to the action of certain chemicals (such as nitrogen mustard). In general, the mutations are of the nature of the loss of a character. It is possible by such means to secure mutant strains of bacteria (as in *Neurospora* mentioned above) which require for growth the addition of certain nutrients which apparently are synthesized by the normal or parent strain. Such mutant strains are proving valuable in the hands of the biochemist in analysis for presence of certain nutrient materials. An organism which has lost the power to synthesize one of the amino acids essential to its development may be used to analyze both qualitatively and quantitatively for the presence of this amino acid in material under analysis. The techniques may be much simpler than those usually used in chemical analysis.

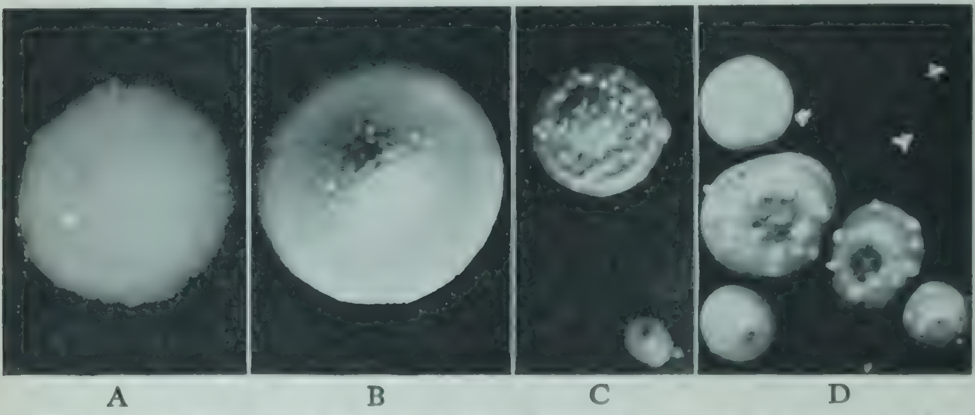

Fig. 8–1. Development of secondary or mutant colonies in colonies of bacteria. A. Colony showing no development of secondary colonies. B, C, D. Colonies of bacteria showing the development of secondary colonies of different types.

Attempts definitely to demonstrate sexual reproduction in the bacteria by microscopic methods have proved in general rather inconclusive. With the newer knowledge gained by use of better techniques such as those of the electron microscope, there is a growing conviction that bacteria have nuclei or at least discrete particles of nuclear chromatin. But actual conjugation of cells, followed by fusion of nuclei, with later reduction division has not been well authenticated.

Is it possible to secure any light on the subject of sexual reproduction by attempting to secure strains with new combinations of characters by trying to "cross" various mutant strains of a species, the mutants differing from each other by several characters? For example, assume that one mutant has lost the power to synthesize several essential compounds, say A, B, and C. A second mutant requires the addition of three different nutrients, say D, E, and F. One may inoculate both strains into a medium capable of growing both. After allowing time for growth, will the pure cultures isolated be identical with one or the other parent, or will there be cultures which show combinations of the characters? The first mutant differs in six characters from the second. It *can* synthesize D, E, and F but not A, B, and C. The converse is true for the second. If cell fusions occur with fusions of nuclei, the chances would be in favor of a redistribution of genes among the resultant cells. One might secure cultures varying from those having no deficiencies to those having any combination of one to six deficiencies. Essentially, results of this type have been secured by Lederberg, who worked with a culture of *Escherichia coli* and succeeded in securing recombinations of characters. In some cases recovery was made of a strain which was as versatile in producing its own needed nutrients as the original strain from which the mutants originated. The kinds of results secured correspond closely with those secured by crossing of higher plants and animals. Apparently, recombinations of characters may occur. However, evidence such as has been outlined does not conclusively prove actual sexual reproduction among the bacteria, but the results are highly suggestive.

Equally surprising and interesting is the fact that somewhat similar results have been secured with virus, particularly with bacteriophage. Mutants (Hershey, Luria, and Delbruek) of a phage were secured which were grown in a suitable bacterial culture, and phages were isolated which had combinations of the characters of the original strain. One may summarize in the words of Pontecorro (1948):

> The simplest working hypothesis is that the underlying mechanism is something of the same kind as that occurring in higher organisms, that is,

that the hereditary particles are organized in bacteria, and perhaps in bacteriophages, in some sort of mechanical arrangement and there are mechanisms for the exchange of these particles between different individual cells, leading to results identical to those of sexual reproduction in higher organisms.

Mutations may occur in many different types of characters, such as shape, size, motility, color, ability to synthesize food or to utilize various nutrients, capsule production, antigenic properties, virulence, and others. Further, if mutants, as noted above, are able to exchange genes in a fashion analogous to sexual reproduction in higher forms through the redistribution of the genes, new combinations of characters may be found in the progeny. One concept to be discussed later relating to the enhancement of virulence in a disease-producing organism is based upon union of two non-virulent forms with resultant combination of characters increasing power to attack.

Dicaryosis and Heterocaryosis. Still another type of complexity must be held in mind in studies of variations in microorganisms.

One great group of fungi (the *Basidiomycetes*) shows an interesting variation in the sexual cycle. The hyphae produced from haploid spores have haploid nuclei and are usually heterothallic, that is, the hyphae are of two types, so-called + and — strains. The cells of hyphae of a + strain coming in contact with those of a — strain will fuse with them (the hyphae *anastomose*), the wall between the cells of the different hyphae stick together, and an opening develops from one cell into the other. The nucleus of one cell passes into the other cell, but does not fuse with the other nucleus. Inasmuch as the nuclei do not unite, the cell does not become diploid in the strict sense, although it now has two nuclei, each with its quota of genes. Such a two-nucleate cell is said to be *dicaryotic*.[2] When the cell divides each nucleus divides separately, and one-half of each nucleus goes to each daughter cell. At a certain stage in the life history of certain cells, the two nuclei do fuse, the cell enlarges (it is termed a basidium) and usually produces four spores, each of which again contains a single nucleus and is haploid. The two nuclei may have different genes; the long multiplication of cells, each with the two nuclei bearing different hereditary characteristics, makes possible a study of the effects upon the plant of two different nuclei growing in the same cellular cytoplasm. The eventual union of the nuclei and the development of the haploid generation bring redistribution of the hereditary characteristics.

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[2] From Greek *di*, two, and *caryum*, a nucleus or kernel.

Even more complex has been found the case with certain molds. Through repeated cell unions there may occur cells which have several to many unrelated nuclei. Furthermore, in some cases multiplication of nuclei within cells may take place without corresponding frequency of cell divisions. In such cases, during cell division the daughter nuclei may be found not to be distributed equally among the daughter cells. Since each nucleus may influence the growth and metabolism of the cell, each new combination may give rise to some detectable variation. Cells containing several nuclei are said to be *heterocaryotic*.[3] The fungi in which these heterocaryotic cells occur are to be differentiated from those forms such as *Mucor* in which partition walls are but rarely formed between cells, the entire mycelium being a much-branched tube (*syncytium*) in whose cytoplasm large numbers of nuclei are to be found, all derived from the parent nucleus or nuclei of the spore from which the mycelium developed.

Microörganisms quite evidently show a capacity for mutation equal and quite similar to that exhibited by higher plants and animals. Furthermore, in many forms, perhaps in most, there are devices which make possible recombinations of characters leading to variation.

Cytoplasmic Inheritance—Plasmogenes. For many years attention was so focused upon the nucleus and its functioning genes in the control of heredity that comparatively little attention was paid to the cytoplasm of the cell and the various bodies contained therein. It has long been known that there are in the cytoplasm of many plants self-duplicating particles, elements visible under the microscope that evidently multiply, which pass to the daughter cells when the cell divides. Such, for example, are the plastids (as the chloroplasts in green plants). Evidence is accumulating that these self-perpetuating units when transferred to the daughter cells are to be regarded as a part of the hereditary mechanism. These cytoplasmic elements or particles have been shown to be important in explanations of certain peculiarities of inheritance in microorganisms; they have been termed *plasmogenes,* that is, particles present in the cytoplasm having some of the characteristics of true nuclear genes.

Are these plasmogenes or cytoplasmic self-perpetuating particles completely independent of the nuclear genes? Their interrelationships are just coming under study through discovery of organisms and characters which are adapted to genetic analysis. A striking example of such interrelationship has been found by Sonneborn in one of the species of the protozoan genus *Paramecium*. It was found that certain strains of this organism produce and excrete into the medium a substance which kills the cells of other strains of the same species. This "killer" substance, poisonous to other strains, has been shown to be produced by some particulate substance present in the cytoplasm of the killer strains but not present in the sensitive strains. These have been named *kappa* particles. They seem to correspond to the plasmogenes of other workers.[4] Cells of different strains of *Paramecium* may be caused to mate. In this process two cells come into contact and unite temporarily, a somewhat complicated set of nuclear changes occurs, with the result that there is an exchange of nuclear material between the cells, and the cells then separate without much apparent admixture of cytoplasm of the two cells. If one of the races that mates in this fashion is a "killer" strain and one a "sensitive" strain, the original "killer" cell remains a killer and the sensitive cell remains sensitive. However, the killer daughter cells are found gradually to segregate into two groups, about one-half retain the killer property unimpaired from generation to generation, the other group in which the ability to produce the killer substance decreases from generation to generation and eventually disappears. Genetic studies have shown that the kappa granules can multiply only when the nucleus contains a corresponding gene. If a cell contains both the cytoplasmic kappa particles and the nuclear appropriate gene, the kappa particles can multiply and every daughter cell can receive its full complement of both gene and kappa. Apparently those cells which lose the killer property in later cell generations have an initial quota of the kappa particles[5] which cannot increase because of the absence of the nuclear gene; at each cell division the number of kappa particles is approximately halved. Within a few generations the number of kappa particles for each cell would be one or none. The *Paramecium* is claimed to be an excellent example of the ability of a cytoplasmic body to multiply under the right conditions, of its ability to determine cytoplasmic inheritance, and of the close interrelationship existing between nuclear genes and the plasmogenes

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[3] From the Greek *heterus,* different or varied, and *caryum,* kernel or nucleus.

[4] Another possible explanation of the phenomenon has been suggested, namely, that the kappa particles are in reality rickettsiae or virus particles parasitizing the cell.

[5] This has been estimated to be about 200-250 particles per cell

Variation Due to Environment. The form of the cells of a microorganism, the colors it produces, whether or not it is motile, the presence or absence of a capsule, the ability to bring about certain chemical changes, will often depend upon the physical and chemical environment of the cells. This variability due to environment will be discussed later under physiology. It is mentioned here to emphasize that it is the ability to produce a certain kind of cell or a particular pigment under a definite environment that is inherited; it is not the shape of the cell nor the pigment.

However, it is not always obvious whether one is dealing in a particular case with the development of a mutant or with a slow response to a change in environment. For example, cultures of certain yeasts are able to ferment the monosaccharide glucose promptly and vigorously in a suitable medium, but when galactose is substituted, fermentation is considerably delayed, though transfers from a vigorously fermenting galactose culture to galactose will produce fermentation promptly. It may be shown that in this case the yeast always has within the cell the enzymes needed to ferment glucose quite independently of the presence of glucose. Such enzymes consistently present irrespective of the environment are said to be *constitutive*. But the yeast is lacking or at least deficient in some one or more of the enzymes needed to ferment galactose; however, in the presence of galactose it is stimulated to the production of such enzymes. Some time is required for these to build up in the cells to a point where vigorous fermentation can occur. Such enzymes which are developed in response to environment are said to be *adaptive*.

This type of slow adaptation is not to be confused with another example of variation, based on mutation. It was earlier noted in this chapter that certain bacteria are known which ferment glucose rapidly and promptly. But in the presence of the sugar lactose, this sugar is not attacked until some time has elapsed, when lactose fermentation occurs. Careful analysis shows, however, that this is not like the preceding, a case of slow formation of adaptive enzymes. Instead, it can be shown that this organism produces lactose-fermenting mutants rarely, it is true; perhaps not more than one cell in a million mutates by the acquisition of the property of lactose fermentation. In this culture the bacterial cells multiply, using nutrients other than lactose present. When a mutation does occur, that cell is able to utilize the lactose; it and its progeny increase rapidly, much more rapidly than the other cells not able to use lactose until the mutant cells are present in numbers sufficient to produce visible fermentation. Examination of the culture from time to time will therefore show an increasing proportion of the lactose-fermenting bacterial cells. But the lactose fermenters are permanently such. When transferred to other media not containing lactose, there is no gradual loss of the power to ferment this sugar. Pure cultures of the remaining dextrose fermenters will be found not to have changed; they still are unable to ferment lactose. There are two distinct strains which if encountered in nature would probably have been described as different species.

Great care is necessary to differentiate between the gradual development in a culture of an adaptive enzyme and the eventual overgrowth of a culture by a mutant which possesses a new constitutive enzyme.

Inheritance of Impressed Variations. Variations in morphology and composition of microorganisms associated with particular environments are frequently observed. The presence of a capsule in a bacterial cell may result from the presence of some particular component of the medium in which the organism is grown. For example, certain bacteria form capsules only in the presence of the sugar sucrose. When such an organism is transferred from a medium in which no capsules are formed to one in which they are produced, no mutation is involved. It is evident that the organism has a heritable capacity to produce capsules under a suitable environment. As a rule, then, modifications due to a particular environment are not inherited. This is sometimes stated as the rule that *acquired characters are not transmitted.*

There are some apparent, perhaps real, exceptions to this rule. One of the best known of these cases is that of certain of the strains of the *Diplococcus pneumoniae* frequently associated with the disease pneumonia. These will be discussed in greater detail later. The important point here is that there are numerous so-called serotypes of these bacteria which can be differentiated each from the other on the basis of serological reactions (see Chapter 31). Study has shown that these differences are due to the ability of each strain to produce certain complex chemical compounds which are characteristic and whose presence can be detected in the laboratory by suitable tests. The ability to produce certain of these substances (so-called specific antigens) seems to be bound up in the cell with the presence of certain definite nucleic acids. Avery (1944) showed that from "Smooth" pneumococci (Type III) may be extracted certain nucleic acid molecules which when brought into contact with "Rough" (Type II) pneu- nococci are taken up and render the latter able to produce thereafter the antigenic substance of Type III. In other words, if this ucleic acid (or its compounds) is extracted from one strain of the acteria and placed in contact with another strain not possessing it, he latter strain may be shown to have acquired some of the special erologic characteristics of the first. The change is relatively a permanent one; by this treatment one strain has been so modified as to ave taken on the characteristic of the other. Apparently, by the addition of this material the formerly deficient strain has acquired the power to synthesize more of the component having the specific antigenic character. In such a case we apparently have an exception to the rule that changes in composition impressed by environment are not inherited. As stated by Burnet (1945), "The nucleic acid molecules so taken up appear to be incorporated into the genetic mechanism and confer an inheritable capacity on the organism to produce Type III polysaccharide. Subject to modifications in the light of future work, the only straightforward interpretation of the phenomenon is that the S(mooth)-R(ough) mutation is a result of the loss of a certain type of nucleic acid molecule from the controlling genetic mechanism." Although much study has been devoted to this phenomenon, no completely adequate explanation has been developed.

Variation in Colony Characteristics. When bacteria are grown in a non-liquid environment, or medium, where the cells cannot diffuse freely, they produce masses of organisms or colonies which are visible to the eye. The characteristics of these colonies will be discussed in greater detail under the heading of observations of cultural characters. To be noted at this point, however, is the fact that any heritable variation or mutation in an organism may give rise to a readily visible difference in the appearance and characteristics of the colony formed. Many species of bacteria, for example, may produce a colony which is comparatively smooth upon the surface, but under suitable conditions colonies of another type which are relatively less smooth may be formed. These are the so-called *smooth* (S) and *rough* (R) phases. These changes in colony characteristics not infrequently are associated with other changes as capsulation, motility, or even disease-producing power or virulence. Terms that are commonly used in the literature of bacteriology to characterize the organisms producing these colony variants are rough, smooth, mucoid, and matt. In general, colonies produced by organisms from one type will continue to produce colonies of the same type; the change from one type to the other has some of the characteristics of a mutation. However, in some cases, at least, the transformation is one which may proceed in either direction, from smooth to rough and vice versa.

Cyclical Changes in Microorganisms: Asporogenous Mutations. Many microorganisms show differences in morphology dependent in part upon the age of the cells. For example, a rod-shaped bacterium when grown in a suitable culture medium may develop in the form of long chains of cells. After a time, however, each cell produces or is transformed into an endospore. There is good evidence that this tendency to sporulate is in part the result of some environmental change, probably a change produced by the organism itself in its growth. When the spores are placed in a suitable environment they are transformed into vegetative multiplying rods. If the vegetative cells are constantly supplied with a fresh medium so that there is the maintenance of a uniform environment, the multiplying vegetative stage may be extended indefinitely. This alternation of vegetative and spore stages does not involve mutations or hereditary change. Occasionally, however, cells in the vegetative stage may mutate by complete loss of power to produce spores and the development of a nonsporulating race. Similar mutations may occur in the fungi and the yeasts leading through a mutation to a permanently asporogenous race. In a fungus such as one belonging to the *Ascomycetes* this might involve the loss of the ability to produce ascospores, but not change the power of producing the asexual spores or conidia. It has already been noted that in the genus *Aspergillus* some species readily produce sexual spores (ascospores), but the formation of ascospores has never been observed with other species. The great subdivision of the fungi, the *Fungi Imperfecti*, are so-called on the assumption that they have lost the power of sexual spore production. In a few cases even the ability to produce asexual spores has been lost; there are fungi known in which the mycelium is sterile (*mycelia sterilia*). It is self-evident that mutations which involve the suppression of one of the cyclical stages in the development of an organism make special problems in determining relationships and in classification. However, the same is to be found with plants of all groups. Among the higher plants, the banana, the navel orange and the Lombardy poplar do not produce seeds, but they are placed systematically in their right relationships to related seed plants on the basis of other characters.

Isolation of Mutants. Most mutants of microorganisms disappear because they are not adapted to their environment. This is particularly true of physiological mutants. For example, mutants are produced which have lost the power to produce some substance essential to the growth of the cell. If this particular substance is not present in the medium, the cell will cease growth and eventually die. It is important that methods of isolating and growing these deficient mutants be developed, for such mutants have become increasingly significant in studies of cellular physiology and in biochemical analysis. Many methods have been developed. One may be illustrated. The antibiotic penicillin is known to injure or destroy growing cells, but resting cells may be relatively little injured. Davis has shown that if to an actively growing culture of the bacterium *Escherichia coli* there be added the minimum amount of penicillin that will destroy the growing (normal) cells, the resting (deficient) cells are not injured and will grow if they are transferred to a medium which contains the substance which the organism has lost the power to synthesize. If radiation is used to produce mutations, isolation by this means is to be attempted only after there has been a sufficient time lapse to allow the deficient cells to become fully dormant.

CHAPTER 9

Distribution of Microorganisms

Where Microorganisms Are to Be Found. Microorganisms are so widely and generally distributed that it is perhaps easier to enumerate the places where they are not likely to be found than where they are usually present. They are not commonly found at great altitudes in the air, at considerable depths in the earth, or in the healthy normal tissues of animals and plants. Otherwise they are ubiquitous wherever the temperature is not so high as to destroy life, or chemical or other agencies do not interfere. They are abundant in the soil and air, in most surface water, in the depths of lakes and oceans, in certain foods, in decaying organic matter of all kinds, on the skin, and within the intestines of man and animals. They will be found growing practically wherever the conditions are favorable for life, that is, wherever there is assimilable food, or where suitable environments have enabled them to produce spores, resting cells, or cysts relatively resistant to drying, low temperatures, and similar unfavorable conditions. These spores persist for long periods of time and may be found viable when placed under favorable environment.

Microorganisms of the Soil. Bacteria are very abundant in most soils. A rich garden soil will contain from a hundred thousand to as many millions per gram. In acid moor or peat soils, in soils containing an excess of alkali, and in poor sandy soils, they are much less numerous. These bacteria perform many functions in the soil, fitting it physically and chemically for the growth of higher plants. Except for the soil bacteria, higher plant life could not long persist. They decompose organic matter directly and the minerals of the soil both directly and indirectly, converting these soil constituents into compounds that may be taken up by the roots and assimilated by green plants. Some forms take up atmospheric nitrogen which is ultimately used by other plants. Bacteria capable of producing disease in plants or animals are occasionally found in the soil.

Molds and other fungi are also quite abundant in soils. In some, as in those of forests, they may bring about decay even more efficiently than do bacteria. The decomposition of cellulose and woody tissues is in large part the work of molds and related fungi.

Yeasts are relatively infrequent and unimportant in the soil. This does not mean that they are often entirely absent, for they usually may be demonstrated by appropriate cultural methods.

Microorganisms in Water. Bacteria are commonly present in most waters, yeasts and molds much more rarely. Water from deep wells, such as the artesian wells of some districts, and from some springs may be entirely free from bacteria. Most well waters contain from a very few to several hundreds of bacteria per cubic centimeter. Lakes and ponds not polluted with sewage have about the same number. Streams usually have more. Sewage and heavily polluted water may contain thousands or millions of bacteria per cubic centimeter. Sewage containing industrial wastes from creameries and canneries because of the relatively high content of carbohydrates may have numerous yeasts and molds. Domestic sewage usually contains bacteriophages of great variety, in fact this material has been frequently used as a source of phages specific for many kinds of bacteria. Although water does not normally contain disease-producing bacteria, it may become polluted with the excretions of diseased animals, or man, and become a dangerous source of infection.

Microorganisms in Foods and Feeds. Food when eaten is rarely free from living organisms. The type of organisms present, whether bacteria, yeasts, or molds, is determined by the chemical composition of the food and by its previous treatment. The organisms of food may be divided into three groups: those that are of benefit in bringing about desirable fermentations, such as those which occur in the preparation of sauerkraut, cheese, or vinegar; those that produce undesirable fermentations and decay; and those that are capable of producing disease. The major problem of food preservation, including refrigeration, canning, and drying, is that of eliminating and preventing the growth of undesirable organisms.

Some healthful foods contain great numbers of bacteria. Clabbered milk, for example, may contain hundreds of millions per milliliter. Foods not freshly cooked usually contain them in considerable numbers, but they are generally not of types that are injurious. Occasionally pathogenic bacteria may be present in the meat of diseased animals or in other food that has been improperly cared for.

Bacteria are abundant in most foods; yeasts may be present in those containing sugar; molds occur upon many fruits and their products and are instrumental in the ripening of certain cheeses.

Microorganisms in Fermentation and Decay. The decomposition of organic matter, of all plant and animal remains, is brought about by the activity of microorganisms. Proteins are usually broken down by bacteria, although molds are occasionally important. Complex carbohydrates, such as starch and cellulose, are commonly attacked by molds and by a few bacteria; the simpler carbohydrates are fermented by yeasts, molds, and bacteria, and the fats by bacteria and molds.

Microorganisms of the Body. Since the body is constantly in contact with substances covered with bacteria, the skin harbors many organisms; and as food is not usually sterile when eaten, the alimentary tract (particularly the intestines) contains bacteria in large numbers. The intestines soon come to possess what may be regarded as a normal flora of bacteria; in fact, the bacteria and protozoa are apparently essential in the digestive processes of many animals. In herbivorous animals, for example, they are needed in the digestion of cellulose and other carbohydrates of the feed, but function as well in the production of essential vitamins. Yeasts and molds are much more rarely found under these conditions. The living normal tissues of the body are commonly bacteria-free. Pathogenic bacteria, however, may break down the barriers to invasion of the body and produce disease. The body excretions, particularly the feces, contain bacteria in enormous numbers; in man, as much as twenty-five per cent by weight of the latter may be bacterial cells. Many insects develop special organs in which microorganisms (usually bacteria or yeasts) are always present, probably of significance in some way to the animal.

How Microorganisms Are Scattered. Those bacteria that possess organs of motion can swim to some distance from their origin under suitable conditions. Convection and other currents in water facilitate the distribution of aquatic forms. Molds, and to a lesser degree yeasts and bacteria, may spread by direct growth. The mycelium of some molds, for example, may grow at the rate of an inch or more a day. The most common medium of dispersal is the air. Certain of the molds are particularly adapted to distribution by this means. They send up their erect conidiophores away from the moist substratum on which they are growing and produce their conidia where they are readily dislodged and blown about by the slightest air current. Some even forcibly project their spores into the air. Bacteria and yeasts are not readily blown about until the material in which they have been growing has dried and has been pulverized. In consequence the air on a dry day, particularly if windy, contains many more organisms than when the air is moist. Organisms are carried also by water, as that in streams, and in milk and other fluids. Insects, particularly flies, may transport them in considerable numbers from one place to another.

CHAPTER 10

Sterilization

Sterilization, as the term is used in bacteriology, is that proces whereby any material is entirely freed from living microorganisms It is important as a device for the preservation of certain foods an other organic materials which may readily decompose. In the bacteriological laboratory and in the surgery complete destruction or removal of all organisms is highly important. Since microorganisms ar present upon the skin, in dust, in the air, upon the surfaces of al laboratory tables and apparatus, it is evident that means must b taken to prevent such forms from coming in contact or becoming mixed with the organisms which it is desired to study in pure cultures

Sterilization may be effected either by *physical* or by *chemica* means. Destruction of organisms by chemical agencies is frequentl designated as *disinfection*, and the use of the term *sterilization* limited to their destruction or removal by physical methods. The physica agents most commonly employed in sterilization are heat, filtration and light. For details relative to techniques of sterilization the student should consult a suitable laboratory manual.

Sterilization by Heat. Sterilization may be accomplished by momentary heating to a high temperature (as to red heat) or by a longe exposure to hot air, to streaming steam, or to steam under pressure and for some purposes by the use of temperatures somewhat lowe than the boiling point of water.

Sterilization by the Flame. Small objects that are not easily injured by heat may be sterilized by thrusting them into the flame o the bunsen burner and heating them to a temperature high enoug to destroy any organisms present upon the surface. This ordinaril means red heat. The platinum or nichrome needles commonly use in the laboratory in the transfer of microorganisms from one culture tube to another are generally sterilized in this manner.

Sterilization by Hot Air. Dry flasks, test tubes, petri dishes, pipettes, and similar articles of glass are commonly sterilized in a hot-air oven which is maintained at a temperature of 150° to 170° C. for an hour. Care must be used to see that objects to be sterilized are not packed into the oven so tightly that heat will not penetrate readily to all parts. This method cannot be employed in the sterilization of liquids or of organic substances which may be injured or decomposed by subjecting them to such temperatures.

Sterilization by Streaming Steam. Moist heat is more efficient in sterilization than is dry heat. Streaming steam or live steam is therefore quite effective and can be used for organic substances such as the laboratory media. This type of sterilization is ordinarily accomplished in an Arnold steam sterilizer or some similar apparatus which allows the live steam to come in contact with the material to be sterilized. An Arnold sterilizer consists of a pan partially filled with water, a portion of which is between the layers of the double bottom. This water between the bottoms soon reaches boiling temperature when placed over a flame and is replaced through suitable openings from the water in the pan above as rapidly as it evaporates. The steam arises through the large opening in the center and escapes finally through the openings in the top or around the doors. Live steam (at sea level) has a temperature of about 100° C. A single exposure for fifteen minutes to such a temperature is ordinarily sufficient to destroy all vegetative bacteria. Some resistant spores, however, may remain alive after this treatment. In practice it is customary to heat on three successive days for fifteen minutes each day. This method of sterilization does not subject the constituents of the medium to a high temperature for a long period of time. This process is called *intermittent sterilization* and may be used on certain types of culture media that might be spoiled for use by higher temperatures and pressures. It is not as efficient as heating in steam under pressure.

Sterilization by Steam under Pressure. The apparatus most used for sterilization by steam under pressure is called the *autoclave* or *digester*. It consists essentially of a closed chamber into which steam under pressure can be introduced. For efficient sterilization *all* of the air originally present in the apparatus should be driven out by the live steam; therefore the stopcock on the autoclave must be left open until all the air has escaped. Most modern autoclaves have automatic air escape valves. The pressure of live steam varies directly as its temperature; consequently when one knows the pressure as indicated by a gage, the temperature is readily determined. It may, in fact, usually be read from the gage itself. This is not true, however, of a mixture of steam and air, for such a mixture has a decidedly lower temperature than would be indicated by the pressure. It is customary to sterilize most media at a pressure of fifteen pounds for ten to fifteen minutes. This pressure of live steam gives a temperature of about 121° C. If the material to be sterilized is very bulky (as large flasks filled with media), it is necessary to heat for a longer period of time to make sure that the temperature of the medium has been raised to that of the steam. This temperature of 121° C. is capable of certainly destroying any bacteria that may be present.[1] For sterilizing gelatin it is sometimes necessary to decrease the pressure to ten pounds and expose to this temperature for ten minutes only. As has been mentioned above, some organic substances cannot be sterilized in the autoclave without decomposition taking place.

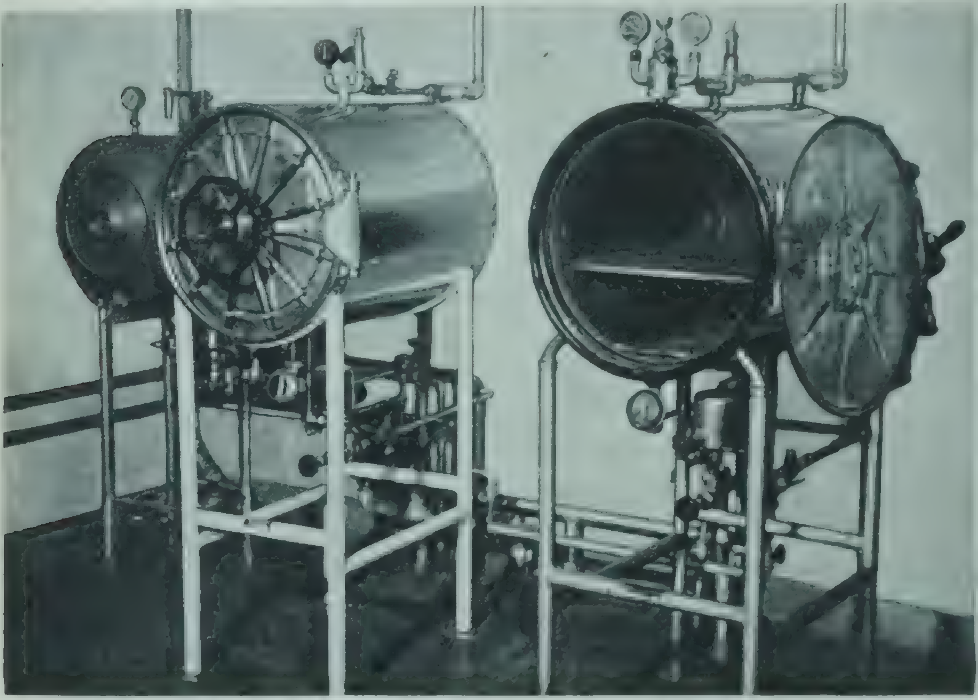

Fig. 10–1. Battery of autoclaves or pressure steam sterilizers in a laboratory. Note temperature and pressure gages.

The common procedure in the commercial preparation of canned foods, such as corn, tomatoes, peas, and beans, is essentially treatment in an autoclave or retort by steam under pressure for varying lengths of time. Pressure cookers are similarly used in preparation and canning of foods in the home.

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[1] The time required to sterilize at any temperature is determined by many factors. These time-temperature relationships are discussed in detail in Chapters 16 and 17.

Sterilization at Temperatures Lower than Boiling Point. It is sometimes necessary to use temperatures lower than the boiling point of water in order to prevent undesirable chemical changes in the medium which is being sterilized. Under such conditions heat must be applied for a longer period (for an hour or more) on each of five or more successive days. Commonly a temperature of from 75° to 80° C. is employed. The sterilization of blood serum is usually carried out in this manner, as it enables one to secure a medium which is semitransparent. When there are many spore-producing organisms present in the medium, it is sometimes impossible to sterilize by this means.

Sterilization by Filtration. Sterilization may be effected by the filtration of liquids or gases through materials that will retain microorganisms.

Filtration of Gases by Cotton. It is customary to plug with cotton the flasks, test tubes, etc., in which bacteria and other microorganisms are grown. In the discussion of spontaneous generation it was noted that air passed through cotton is usually deprived of its dust and microorganisms. It is therefore possible to sterilize media in tubes or flasks that have been plugged in this manner, and, if properly done, no organisms will develop, although the cotton will allow more or less interchange of gases between the inside and the outside. If, however, the cotton plug has become moist or if the medium is stored in a place that is too damp, molds and sometimes bacteria may grow down through the plug and appear on the inside, then, of course, contaminating the contained medium. When properly prepared and cared for, however, cotton plugs are efficient in the sterilization of the gases which enter the flask.

Filtration of Liquids. Many filters constructed of unglazed porcelain, sintered glass, or other porous inorganic materials, which will not permit the passage of microorganisms through them, have been placed upon the market. These are prepared in many sizes and shapes and of many pore sizes. It is, of course, necessary that the vessels used and the filters themselves be sterilized before any attempt is made to remove bacteria by passing liquids through them. Filtration is particularly useful in sterilization when dealing with substances like blood serum which are very easily changed by heat, particularly sera containing antitoxins and all culture media containing toxins. These filters have been advocated for the purification of drinking water. In some cases they are screwed directly on the tap. It is found that in most cases the bacteria do penetrate these filters in the course of time, and such filters therefore must be sterilized at short intervals if they are to remain efficient. Something other than mere pore size of the filter probably determines its efficiency. Adsorption of the bacteria to the pore walls of the filter is undoubtedly an important factor in their removal.

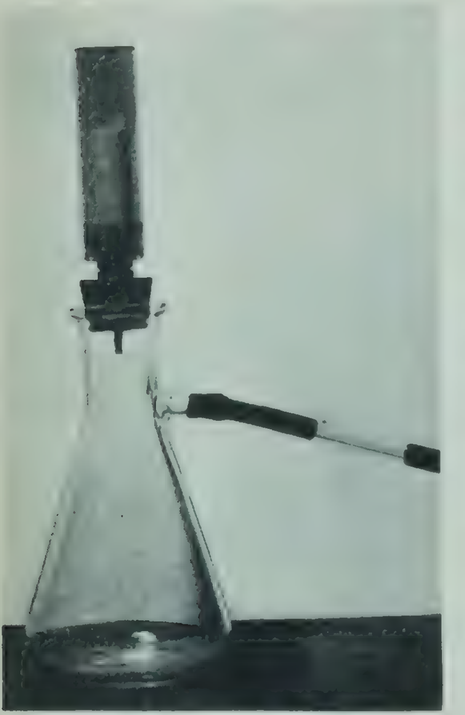

Fig. 10–2. A porcelain filter in operation. The filter, flask, and all connections are sterilized by heat. The tube to the right is connected to a vacuum. The atmospheric and hydraulic pressure force the liquid to be sterilized through the pores of the filter, removing bacteria and larger microorganisms.

Sterilization by Light. It will be noted later that certain rays of light, the blue, violet, and ultraviolet in particular, are destructive to living cells. Advantage has been taken of this fact in the construction of certain types of electric lights for the destruction of bacteria in water. A Cooper-Hewitt mercury vapor lamp with a quartz tube gives out a large proportion of these destructive ultraviolet rays and has been put into practical use for sterilization of water in large quantities. Other types of radiant energy as x rays will also destroy microorganisms, but are not often used in practice for sterilization.

Sterilization by Ultrasonic Vibrations. Methods have been developed whereby sound waves of a frequency far greater than can be heard are used to destroy microorganisms. The cells are caused to vibrate so violently that they are literally shaken to pieces.

CHAPTER 11

Preparation and Uses of Culture Media

Any nutrient or combination of nutrients so prepared as to be directly usable for the growth and cultivation of microorganisms of any kind is termed a *culture medium*. Many media have been found useful, not only to keep the organisms alive and growing, but also to assist in differentiating them. Upon or in media of various kinds the organisms are able to exhibit physiological activities as well as their special morphology and growth characteristics.

It is the purpose of this chapter to describe in somewhat general terms the commoner culture media, their preparation and use, without going into detail. For specific directions for preparation of media a laboratory manual should be consulted. Further, only a few of the most common of the media will be listed. There are in the literatures of bacteriology, zymology, and mycology literally thousands of different media described. In fact, any one doing research in these fields is constantly trying new combinations which will help him to get the needed facts.

SOME GENERAL CHARACTERISTICS OF NUTRIENT MEDIA

A nutrient medium to be useful for the growth of a microorganism must possess certain characteristics: (1) it must be neither too acid nor too alkaline, i.e., it must possess a suitable reaction; (2) it must contain all the kinds of nutrients required by the organism to be grown, and in suitable amounts; and (3) it must in most cases be sterile before use. These characteristics will be considered in order.

The Reaction of the Culture Medium and Its Adjustment. For each kind of microorganism there is a range of reaction of media in which growth will take place; it will not grow in a medium that is too acid or too alkaline. In many cases it is therefore necessary to adjust the reaction of a proposed medium.

There are two principal methods of describing the reaction of a medium. The first of these is noted briefly, as it is principally of historical interest only. This older method is to determine by titration the total amount of acid or base present and to describe the reaction by designating the amount of normal acid or normal base[1] required to bring one hundred milliliters of the medium to neutrality as measured by some indicator. This method was formerly used almost exclusively until it was realized that the growth of microorganisms in a medium depends not only on the *amount* of acid present but upon the *degree to which it dissociates* into hydrogen ions and cations, in other words, upon whether it is a strong or weak acid. Growth is most influenced by the concentration of hydrogen (or hydroxyl) ions in the medium.

The second and more common method of designating acidity is by use of a measure of the concentration (better, the activity) of hydrogen ions, called the pH of the medium.

In a somewhat limited sense, a measurement of the hydrogen ion activity[2] (or pH) is a measurement of that characteristic that we term sourness. In general, the more sour a solution is the higher its concentration of hydrogen ions. One may determine the amount of acid in a solution by titrating against a suitable standard solution, but this does not measure the true acidity. This latter is due to the presence of hydrogen ions which have arisen through dissociation of the acid. Alkalinity, on the other hand, is due to the presence of dissociated hydroxyl ions. The true acidity is determined not only by the amount of acid but also by the kind of acid. Hydrogen ions are dissociated in relatively large quantities by so-called strong acids in solution and in small quantities by weak acids. Equal concentrations of strong and weak acids show therefore marked differences in true acidity, i.e., in their respective hydrogen ion concentrations. In addition, the hydrogen ion concentration of a solution may be modified by substances which change the degree of dissociation of the acid; these are called *buffers*. It is evident therefore that the true acidity ("sourness") of a solution depends on at least three important factors: (1) the amount of acid present, (2) the kind of acid present, and (3) the buffer present.

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[1] A normal solution of an acid is one that contains "the hydrogen equivalent in grams per liter," or practically one gram of acid (replaceable) hydrogen per liter of solution (accurately 1.008 grams per liter). To prepare a normal solution, the molecular weight of the acid is calculated, and, if the acid is monobasic, this amount in grams is made up to one liter of solution. If more than one acid hydrogen atom is present in the molecule, the molecular weight is divided by the number of such atoms. For example, 60 grams of acetic acid (CH_3COOH, molecular weight 60) made to a liter of solution with distilled water is a normal solution. In normal sulfuric acid (H_2SO_4, molecular weight 98) 49 grams are present per liter. Normal alkalies (bases) are those that neutralize exactly equal volumes of normal acids. In descriptions of culture media acidities are usually indicated by the plus (+) sign, alkalinities by the negative (−) sign. A reaction of +1.0 means that in 100 ml. of the medium, there is an equivalent of 1 ml. of normal acid. In the determination of the reaction phenolphthalein is used generally as the indicator.

[2] The influence of the hydrogen (or hydroxyl) ions upon growth of microorganisms is determined by their so-called *activity*, not alone by their concentration. However, for practical use in the bacteriological laboratory, the term hydrogen ion activity and the hydrogen ion concentration can be used interchangeably in most cases without serious error. Strictly speaking, the hydrogen ion activity is mathematically the product of the hydrogen ion concentration and a hydrogen ion activity coefficient. The latter varies with concentration of acid and other medium components. But in dilute solutions such as are usually employed in media the coefficient is nearly equal to one, and hydrogen ion activity and hydrogen ion concentration are approximately equal.

The scale which has proved most logical and useful in designating the acidity of a solution is the pH scale already noted. Its origin and use should be understood inasmuch as it is constantly employed in biological studies.

Chemically pure water is neutral because, when a molecule of water dissociates, it forms one hydrogen and one hydroxyl ion. The numbers of hydrogen and hydroxyl ions in pure water must remain equal. The physical chemist has been able to prove, furthermore, that pure water (and therefore any neutral solution in water) at 22° C. contains approximately one ten-millionth of a gram (10^{-7} gram) of hydrogen ions to the liter.[3] The concentration may be still more conveniently expressed in terms of normality of hydrogen ions. A normal solution of hydrogen ions is one which contains one gram of hydrogen ions per liter. A neutral solution, therefore, is one which has a concentration of hydrogen ions of 10^{-7} normal. Since in a neutral solution there is the same number of hydroxyl ions as of hydrogen ions, the hydroxyl ion concentration must also be 10^{-7} normal.

The chemist has shown that in any dilute aqueous solution the *product of the normality of hydrogen ions by the normality of hydroxyl ions is a constant number* (the so-called *dissociation constant*). The numerical value of this number may be determined by multiplying 10^{-7} (normality of hydrogen ions in a neutral solution) by 10^{-7} (normality of hydroxyl ions in a neutral solution). This product is 10^{-14}. In other words, the product of the normality of the hydrogen ion concentration of a solution by the normality of the hydroxyl ion concentration must always be approximately 10^{-14}. If we know either the hydroxyl or hydrogen ion concentration, we can at once determine the concentration of the other. For example, if we are dealing with a solution having an hydrogen ion concentration of 10^{-4} normal, its hydroxyl ion concentration must be 10^{-10} normal. A scale to designate the acidity of any solution in terms of its hydrogen ion concentration may be arranged as follows:

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[3] Dissociation is increased by rise in temperature.

$$- - - 10^{0}\ 10^{-1}\ 10^{-2}\ 10^{-3}\ 10^{-4}\ 10^{-5}\ 10^{-6}\ 10^{-7}\ 10^{-8}\ 10^{-9}\ 10^{-10}\ 10^{-11}\ 10^{-12}\ 10^{-13}\ 10^{-14} - - -$$

On this scale it will be noted that the larger the numerical value of the exponent, the smaller the hydrogen ion concentration. Inasmuch as this method of statement is somewhat cumbersome, it was suggested by Sörenson that the exponents be used to indicate the scale, dropping the negative signs. This gives the scale:

– – 0, 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14 – –

This series of numbers constitutes the pH[4] scale. This abbreviation comes from the fact that an electrometric method of measuring hydrogen ion concentration is frequently used in which the pH is determined from the electrical potential produced by the hydrogen ions, i.e., the p stands for the German "Potenz," meaning power or exponent.

A solution having a pH of 0, for example, would have a normality of hydrogen ion concentration of 10^{0} or 1, one having a pH value of 7 would have a hydrogen ion concentration of 10^{-7} normal, that is, it would be neutral. It is evident, therefore, that the *smaller* the number on this scale the *higher* the hydrogen ion concentration, that is, the greater the actual acidity, and the larger the number the greater the actual alkalinity.

One characteristic of this scale must be kept in mind. Each number represents a hydrogen ion concentration ten times that of the number to the right. For example, the hydrogen ion concentration of a solution which has a pH 6 is ten times that of one with pH 7.

Accurate determination of the pH of a medium (or of any solution) is usually made electrometrically by means of an electrode, usually of glass. But the degree of precision required in determination of the pH of a medium in the bacteriological laboratory is usually satisfied by the use of indicators which are more easily employed than are electrodes.

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[4] Technically the pH value is the logarithm of the reciprocal of the hydrogen ion concentration. However, this is simply another way of saying that the exponents of the hydrogen ion concentration as noted in the scale above are used, changing the negative signs to positive.

Indicators are chemical substances, usually dyes which change color with changes in hydrogen ion concentration, that is, an indicator is one color in a certain range of pH values or hydrogen ion concentrations, and another color in other ranges. For example, one of the indicators longest used is litmus, a colored compound which is extracted from a species of lichen. It is lilac in color at true neutrality, that is, at pH 7. At pH 8, that is, in a more alkaline solution, it is blue. At pH 5, that is, in a stronger concentration of hydrogen ions, it is red. Other indicators change color at other pH values. For example, phenolphthalein is colorless in all hydrogen ion concentrations having a pH value less than 8.2; in more alkaline solutions it is red. Many other indicators are known which change color at other points in the hydrogen ion or pH scale. This change of color is not complete at a particular pH value. In adding alkali, for example, to an acid solution containing litmus there is not a transformation of red into blue at a particular or definite pH. The color changes gradually with the addition of a base, there is a *range* of pH values over which the color change occurs. By the use of standards whose hydrogen ion concentration is known it is possible to determine approximately the hydrogen ion concentration of any material which it is desired to test by the use of the intensity of color of appropriate indicators. This will be discussed in Chapter 14.

Most bacteria grow well in media having a pH between 7 and 8. The pH of blood is usually about 7.35; this, then, is the hydrogen ion concentration commonly found most useful in cultivating many species of pathogenic bacteria. Media intended for the cultivation of yeasts and molds are usually much more acid, with a pH of from 3 to 5. Since the ingredients used in making media are frequently acid, it is necessary to add alkali, usually potassium or sodium hydroxide, until test shows that the hydrogen ion concentration has been satisfactorily corrected. The actual adjustment of the reaction is accomplished by determining how much of a standard base (as $N/_{20}$ NaOH) is required to bring a measured portion of the medium to the desired pH as shown by the color of a suitable indicator. From this the amount of standard alkali required to adjust the reaction of the entire amount of medium may be calculated.

The hydrogen ion concentration of a medium or solution depends not only upon the actual concentration of the acid itself but also upon the concentration of substances which are termed *buffers*. A buffer is any substance in a solution which tends to prevent marked changes in hydrogen ion concentration upon the addition of bases or acids. Certain salts, particularly the phosphates and carbonates and many organic substances, such as the amino acids and peptones, act as buffers. For example, the addition of a small amount of an acid to distilled water will give a marked change in the hydrogen ion concentration, but the same amount of acid added to solutions containing considerable amounts of buffer may result in very slight differences in hydrogen ion concentration. Inasmuch as microorganisms are usually much more sensitive to differences in hydrogen ion concentration than they are to the total amount of acid present, well-buffered media are commonly used for their growth.

Nutrients Used in Preparation of Media. Microorganisms differ greatly in their food requirements. This will be more evident from the discussion of microbial physiology in Chapter 19. Some microorganisms can manufacture all their food needs from relatively simple inorganic compounds, using water, carbon dioxide, oxygen, ammonia, or nitrates and certain salts. Such organisms require a source of energy, light in some cases (with *photosynthetic* organisms) or some readily oxidizable substance as hydrogen sulfide (with *chemosynthetic* organisms). Media for growing such organisms may be very simple, containing no organic compounds.

Most microorganisms require media which contain organic compounds. Here again there is great variety as to requirements. Some need only that the carbon be organic (sugars or organic acids, for example) and can utilize nitrogen in the form of ammonium salts or of nitrates. Such organisms are evidently able to synthesize all the amino acids and other nitrogenous compounds required for production of cell proteins and nucleic acids.

Media to be suitable for growth of other organisms must contain organic nitrogen compounds, such as certain amino acids. In some cases relatively complex compounds may be required, such as hemin from blood.

Many organisms must also have certain vitamins or so-called growth factors present. What these are will be considered later.

Still other organisms have thus far been grown in the laboratory only in the presence of living cells which they can parasitize. Many protozoa, for example, actively ingest and digest bacterial and other minute cells, and a suitable medium for their cultivation must contain such cells. Each bacteriophage must be supplied with living cells of the particular kind of bacterium parasitized. Viruses have not been cultivated apart from living cells. The same is true of the rickettsiae and even of some of the bacteria such as the treponema of syphilis and the bacterium of leprosy. Much use has been made of chick embryos in incubating eggs for cultivation of many obligate parasites.

Culture media obviously must be of many types; in fact, any one doing research with microorganisms is constantly devising new media to enable him to get at their exact nutritional requirements.

Media may be used for many different purposes. In general, they may be classified into those which are useful in cultivation of many kinds of organisms and for maintaining them alive for considerable periods of time. Some media are highly selective, they favor the growth of some organisms and inhibit the growth of others. Other media are designed to enable the observation of certain physiologic characteristics of an organism, such as ability to produce gas from a certain sugar. Media are also designed for following through the successive changes which an organism may produce in a compound, i.e., for the determination of the steps in the process. Media are also planned to show the effects of various environments, both chemical and physical, upon growth. Other media are compounded for large-scale production of chemicals, such as antibiotics formed by certain organisms.

Among the ingredients commonly used in preparing culture media the following may be listed.

Proteins. Many kinds of proteins have been employed in preparation of culture media. A protein is a compound of very high molecular weight which when hydrolyzed by proteolytic enzymes, acids, or alkalies, breaks down into smaller molecules, the proteoses, the peptones, the peptides, and finally the several amino acids. Among the proteins commonly used are blood serum, egg white and egg yolk, casein, and gelatin.

Proteoses are prepared by hydrolyzing proteins; they differ by being soluble in hot water. They are useful in cultivation of some bacteria.

Peptones are the product of further hydrolysis. They are complex in structure, but have smaller molecules than proteoses. Peptones are not precipitated by saturated ammonium sulfate as are the proteoses. Peptones are among the commonest ingredients of culture media.

Peptides, which are comparatively short chains of amino acids, have been used occasionally to study the specific enzymes produced by microorganisms which are capable of splitting certain linkages.

Amino acids are essential in the determination of the exact nutrient requirements of microorganisms. They are usually present in the commercial peptones.

Carbohydrates of many kinds are employed in media, principally the pentoses, xylose and arabinose; the hexoses, glucose, fructose, galactose, and mannose; the disaccharides, maltose, sucrose, lactose, cellobiose, melibiose, and trehalose; the trisaccharide raffinose, and numerous polysaccharides as starches, dextrins, mannans, dextrans, galactans, and the related pectins, the hemicelluloses, and the celluloses.

Organic acids such as those of the fatty acid series, particularly acetic, propionic, and butyric, hydroxyacids such as lactic, dicarboxylic acids such as oxalic, fumaric, and malic, and tricarboxylic acids such as citric are sometimes useful.

Alcohols such as methanol, ethanol, and propanol, glycerol, dulcitol, and mannitol are often included.

Inorganic Salts and Compounds. Usually these are incorporated because of the usefulness of the several ions, such as calcium, potassium, sodium, iron, manganese, sulfate, chloride, and carbonate. In some cases, as with sulfur, hydrogen sulfide, ferrous iron, the organism may bring about oxidation and utilize the reaction as a source of energy. In most non-synthetic media the various components such as peptone have sufficient amounts of these salts to provide what is necessary for the growth of microorganisms. In preparation of synthetic media great care must be used that the several required inorganic ions are present.

Growth Factors and Vitamins. Many microorganisms require the presence of certain growth factors (or vitamins) in order to develop normally. Their function will be discussed later. Among these are *hemin* (x-factor) need for growth of species of the genus *Hemophilus*, thiamin, riboflavin, nicotinic acid, pyridine nucleotides (v-factor), pyridoxine, pantothenic acid, biotin, para-aminobenzoic acid (p-factor), inositol, and others.

Plant and Animal Extracts. Water extracts prepared from various animal and plant materials have been found to have a stimulating effect upon the growth of many organisms, hence the common use of meat infusions, beef extract, liver extract, heart muscle infusion, tomato juice, yeast extract, yeast autolysate, legume extracts, and many others. In general they owe their ability to stimulate to their content of various growth factors. One plant extract that has come into common use is *corn steep liquor,* widely employed because of the stimulating effect of certain of its constituents on the growth of microorganisms used in the manufacture of antibiotics. It is a byproduct of the process of the wet milling of corn (maize) in which the grain is softened by soaking in water usually containing sulfur dioxide; the corn steep liquor drained off is found to contain many substances extracted from the grain.

Other Nutrients. The above list of nutrients is by no means complete but illustrates the complexity which may enter into the preparation of adequate media for the cultivation of bacteria, yeasts, and molds.

Principal Types of Media. A discussion of all the types of media that have been proposed and used for special purposes is not practicable here. There are thousands of such media. There are a few, however, that have proved suitable to a wide range of organisms, and these may be considered briefly. Other types of media used for particular purposes will be noted in connection with the discussion of the specific organisms or uses.

The student should know something of the composition and methods of preparation of the media most commonly employed in the laboratory. However, he will find that the problems of media preparation have been greatly simplified by the manufacture commercially of dried media of many different kinds. These need only to have water in suitable amounts added, the solids dissolved, and the medium sterilized to be ready for use.

Media may be grouped under two general headings, those requiring living cells or tissues to be parasitized by the organisms to be cultured and those having no such requirement. The latter only will be considered at this point. The media which do not contain living cells or tissues may again be divided into two groups, *non-synthetic* and *synthetic.* A *non-synthetic medium* is one in which the exact chemical composition of each of the constituents is not certainly known, frequently even the exact percentage of these constituents is uncertain. Such media, for example, are potato and milk. In a *synthetic medium,* on the other hand, only pure chemicals of known composition are used. The use of a synthetic medium is distinctly advantageous when it is desired to determine certain physiological characteristics of organisms. A medium in some cases may be non-synthetic in part, but the addition of certain chemicals, as the sugars, may make possible the recognition of fermentative capacity of the organisms being studied. Media may also be divided into three classes on the basis of consistency: *liquid media, liquefiable solid media,* and *non-liquefiable solid media.*

Liquid Media. The most commonly used of the non-synthetic liquid media are *beef broth* or *bouillon, peptone solution, milk, blood serum,* and *beerwort,* and their various combinations and modifications.

NUTRIENT BROTH OR BOUILLON. This is made in one of two ways: either directly from meat or from meat extract. In the preparation of the first type, meat infusion, 500 grams of lean minced beef is soaked in water (preferably distilled water) in the ice chest for twenty-four hours, squeezed through cheesecloth by means of a meat press, and the filtrate made up to one liter. To this is added one per cent (10 grams) of a standard peptone, and one-half per cent (5 grams) of sodium chloride. "Peptone" is a mixture of proteoses, true peptones, and some amino acids derived from the digestion of protein by pepsin; it may be considered as partially digested animal or plant protein. It differs from the protein from which it is derived by being soluble in boiling water. These materials are brought into solution, the reaction adjusted, boiled for five minutes, filtered, and sterilized.

Preparation of bouillon from meat extract is very similar except that beef extract (3 grams in a liter of water) is used in place of the beef infusion. Broth is frequently modified by the addition of sugars or glycerol.

Peptone solution is prepared by dissolving peptone in water.

MILK. Milk is a suitable medium for the growth of a great number of organisms, as it contains carbohydrates, fats, and proteins, and, when fresh, possesses a suitable reaction. It is usually sterilized in the autoclave. Separated milk should be used, certified milk if it can be procured. If separated milk cannot be secured, the milk may be pipetted from below the cream layer of a flask kept in the refrigerator for twenty-four hours. Milk is commonly tinted blue by the addition of sterile litmus or bromcresol purple to act as an indicator of the development of acid.

BLOOD SERUM. Sterile liquid blood serum, alone or mixed with broth, is sometimes used as a medium. It is difficult to sterilize such serum after removal from the body without causing it to coagulate, hence, the necessary precautions to insure sterility and prevent contamination are taken at the time the blood is drawn. It is allowed to stand in a sterile vessel until it clots, then the serum is pipetted off.

BEERWORT. Unhopped beerwort is a very useful medium for the cultivation of yeasts, molds, and some bacteria. It is prepared by soaking malted grain, usually barley malt in water. The starch of the grain is changed to sugar by the diastase present, and this malt sugar (maltose), together with certain of the protein constituents of the grain, passes into solution. It should be boiled and filtered to remove all substances coagulated by heat before being placed in test tubes and sterilized. The medium may also be prepared from desiccated malt extract.

Liquefiable Solid Media. Liquid media may be made solid by the addition of suitable amounts of gelatin or agar. Gelatin is prepared by boiling bones, joints, and tendons. When dissolved in water it solidifies or gelatinizes when cool and remains liquid when warm. The addition of gelatin to bouillon or beerwort transforms them into media which are liquid when warm and solid when cold. Chemically, gelatin is a protein which upon hydrolysis yields amino acids. Its use in culture media is somewhat limited by the fact that it liquefies at blood heat and can in consequence be used as a solid medium for cultivation of organisms only at lower temperatures. Many kinds of organisms when grown in a medium solidified by gelatin produce a digestive enzyme, gelatinase, which liquefies the medium, a characteristic useful in differentiation.

Agar (sometimes called by its Malayan name of agar-agar) is a corbohydrate-related substance prepared from various species of marine red seaweeds (*Rhodophyceae*). Agar is one of several phycocolloids, so-called because they are derived from seaweed and because they form a colloidal solution in hot water and produce a gel on cooling. Agar was originally secured largely from Japan, but is now produced in the United States from the alga *Gracilaria confervoides* in North Carolina and from *Gelidium cartilaginosum* in California. Agar when hydrolized (as by heating with acid) breaks down into the sugar galactose and sulfuric acid. It is apparently made up of a long chain of galactose units esterified with sulfuric acid and normally occurs as the calcium salt (calcium agarinate). Agar is soluble in hot water. A 1 per cent solution sets to a jelly at temperatures of 35° to 45° C., although the gel does not melt until a temperature of from 85° to 100° C. is reached. There are relatively few microorganisms which attack agar, consequently it is widely used for the preparation of solid media containing a great variety of nutrients. It is neutral in reaction, therefore it does not usually modify the acidity of a solution to which it is added. In media near a pH of 7 it is resistant to heating in the autoclave. If in an acid medium, it may hydrolyze when heated and fail to become solid when cooled.

NUTRIENT OR BOUILLON GELATIN. Nutrient gelatin is prepared by the addition to bouillon of 10 to 15 per cent of gelatin specially manufactured for use in nutrient media. It is customary to cool the medium down to a temperature below 60° C. after the gelatin has gone into solution and then add the white of an egg. The medium is then heated until the egg white has completely coagulated. This carries down many of the finer particles that have been in suspension and renders the medium clear. Gelatin may be sterilized in the autoclave, provided a pressure of not more than ten pounds for fifteen minutes is used. The application of too much heat, or heat applied for too long a time, will destroy the ability of gelatin to solidify when cooled. Gelatin is usually somewhat acid; it is therefore necessary to adjust the reaction after its addition to the medium.

NUTRIENT OR BOUILLON AGAR. Agar (1.5 per cent) is added to nutrient bouillon and heated until it has passed completely into solution. Frequently laboratory workers prepare a bouillon or broth of double strength and dissolve the agar needed in an equal amount of water by means of the autoclave, and then mix the two solutions. Nutrient agar is one of the media most commonly employed in the laboratory.

SUGAR GELATIN AND SUGAR AGAR. Gelatin and agar may be modified by the addition of various sugars, or in some cases by the addition of both sugar and litmus, or other indicator.

GLYCEROL GELATIN AND GLYCEROL AGAR. The addition of glycerol to the gelatin or agar renders them much more suitable for the cultivation of a few pathogenic bacteria.

BEERWORT GELATIN AND BEERWORT AGAR. Beerwort may be transformed into a liquefiable solid medium by the addition of either gelatin or agar, as has been described. These media are particularly valuable for the cultivation of yeasts and molds. It is not customary to neutralize the beerwort, as both yeasts and molds flourish better upon a medium which is somewhat acid in reaction.

Non-Liquefiable Solid Media. The non-liquefiable solids most commonly used are potato, coagulated blood serum, and coagulated egg. In addition certain other materials are sometimes used, such as carrots, peas, beans, and corn meal.

POTATO. Sterilized potato was one of the first media introduced into the laboratory. It is customary to use cylinders cut from the potatoes by means of an apple corer or special potato borer. These cylinders are then divided longitudinally by a diagonal cut and placed in running water for a few hours. This treatment prevents them from turning black when sterilized later. They are then placed with the slope up in special potato tubes or in large test tubes having a small bit of cotton in the bottom. The bulb of the special potato tube should be entirely filled with water; in the other tubes the cotton should be saturated. This prevents the potatoes from drying out too rapidly. Sterilization is effected in the autoclave at fifteen pounds pressure for fifteen minutes. Potatoes are more difficult to sterilize than some other media because they ordinarily are covered with spore-producing bacteria of a particularly resistant type.

CARROTS, BEANS, etc. Carrots are sometimes prepared in the same manner as outlined above for potatoes. The sterilized pods of green string beans are likewise useful for the cultivation of certain molds. Starch paste, moistened corn meal, bread crumbs, etc., are sometimes utilized for the study of some organisms having special growth requirements.

BLOOD SERUM. Blood serum, either alone or mixed with bouillon or glycerol, is coagulated in a slanting position in test tubes by means of heat. Such media are particularly useful in the cultivation of certain pathogenic bacteria. A satisfactory substitute for cultivating some bacteria may be prepared by mixing the white and yolk of an egg and sterilizing in the same manner.

Synthetic Media. A medium whose exact chemical constitution is known is often necessary in order that the exact nutrient requirements of microorganisms may be determined and the products of their activity and the course of their metabolism studied. Many kinds of synthetic media have been proposed for definite specific purposes. It is essential in such a medium to see that certain salts are present, usually sodium chloride with traces of calcium, magnesium, potassium, and phosphorus salts as well. Some of the bacteria develop only upon media having some protein material present or which contain some specific amino acids and growth accessory factors. The majority will grow, provided there are suitable sugars or glycerol in addition to salts such as those of ammonia or of nitric acid, capable of furnishing nitrogen. Synthetic media may be solidified by the addition of agar.

CHAPTER 12

Pure Culture Methods in Bacteriology

Pure and Mixed Cultures. Any growth of organisms on laboratory media is termed a *culture*. Such a growth upon a solid medium, such as nutrient agar, that has originated from a single organism or spore or from a clump of organisms is called a *colony*. A culture in which only one species of organism is present is said to be *pure;* one in which several are present is *mixed*.

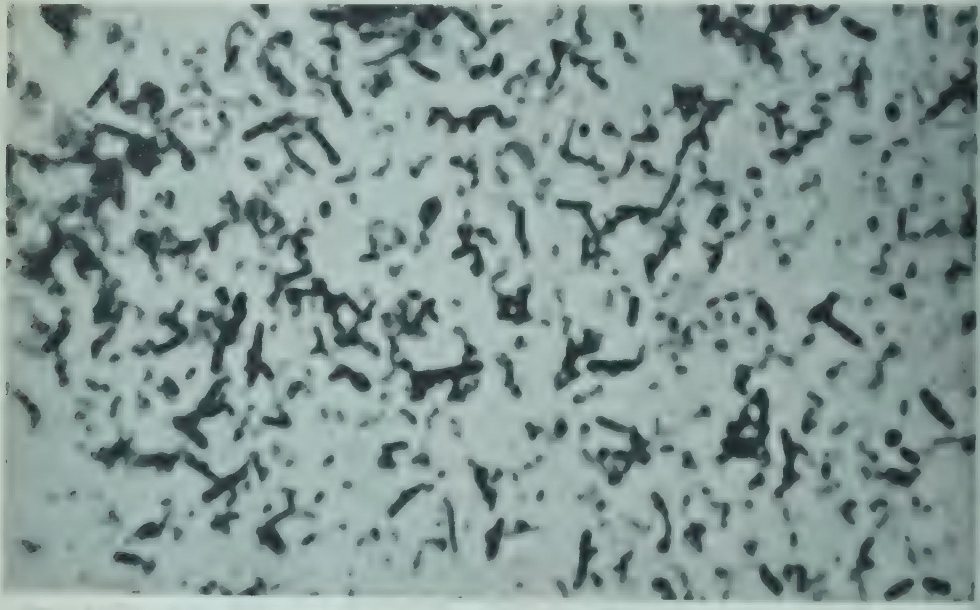

Fig. 12–1. Photomicrograph of a stained preparation showing a mixture of different kinds of bacteria such as is found in many decaying materials. In order to secure pure cultures of the several kinds of bacteria here seen, it would be necessary to separate the cells in some suitable medium so that each may grow into a colony consisting of the progeny of a single cell.

An organism ordinarily must be in pure culture before it can be studied satisfactorily, or at least before its distinctive physiological characteristics can be determined. There are occasions where organisms may be found in pure culture in nature but this is unusual; several or many species commonly occur together in most situations. Methods for resolving such natural mixtures are therefore of fundamental importance. Each kind of organism must be separated from the others and grown in pure culture. Many methods have been devised, some of the more important of which will be briefly noted.

Methods of Transfer. Microorganisms are commonly transferred from one medium to another for cultivation, or to a microscopic slide for examination, by means either of a glass pipette or of a wire (platinum or nichrome) needle or loop. The pipette (sterilized in the hot-air oven) is used with liquids when it is necessary to transfer a measured quantity. The wire needle may have a glass or a metal handle. The wire is sterilized by heating to a glow in the flame of the bunsen burner. After cooling, it may be touched to the growth in a culture. A sufficient number of cells will usually adhere to enable one to make a planting upon another medium. For transferring small quantities of liquids a loop is made in the end of the wire.

Methods of Securing Pure Cultures. Many methods have been devised and used for securing pure cultures of microorganisms. New methods must constantly be devised for special cases. Some of the more common are noted briefly.

Direct Transfer. Under some natural conditions bacteria, yeasts, and molds may be found in pure culture, and transfers can be made directly to a suitable medium. For example, the aerial conidia produced by many molds are comparatively free from contaminations. The platinum needle will frequently transfer the one type of cell. In certain diseases of man, as typhoid fever, and of animals, as anthrax, the bacteria characteristic of the disease are found in the blood. If infected blood is removed with adequate precautions to prevent contamination, as by use of a sterile syringe and needle, it may be transferred to a suitable medium and a pure culture secured.

A special case of direct transfer is *single cell isolation.* Obviously, if a single viable cell may be picked up and transferred to a sterile medium on which it will grow, a pure culture will develop. However, the cells of microorganisms are so small that such direct selection and transfer must be performed under the microscope. There have been developed several kinds of *micro-manipulators* by means of which it is possible under direct observation to select and pick up a single cell and place it in an appropriate medium for growth. The mechanism is one by means of which a micropipette may be moved about accurately and may be filled and emptied. This technique is of special value when one wishes to secure a pure line of an organism as in studies of variation.

Single cell isolation is of particular significance in certain types of inheritance studies with yeasts and fungi. For example, it may be desired to separate the four spores produced in a single ascus of a yeast and to secure a culture from each. Much information of genetic significance has been secured by such separate culturing of all eight spores in the ascus of the mold *Neurospora*.

Isolation by Streaking. A mixture of various organisms, if smeared over the surface of a solid culture medium in successive streaks, will not be uniformly distributed. The various bacteria will grow in a mass where most thickly inoculated; they will develop distinct colonies where fewer are planted. Microscopic examination will often show some of these to be made up of a single species of organism. Transfers may then be made from such colonies to other media and a pure culture may be secured.

Isolation by Dilution. This method was the first employed in the separation of mixed cultures, particularly in the early study of yeasts. Decreasing amounts of the original inoculating material are added to a series of flasks or tubes containing a liquid medium. This is usually accomplished by adding a measured amount, one milliliter, for example, to a tube of a suitable nutrient solution, the same amount from this tube is inoculated into a second, from this to a third, and so on. It is evident that, if a sufficient number of dilutions is made, some of the higher dilutions will contain no organisms and in consequence will show no growth upon incubation. Some of those showing growth will, upon microscopic examination, be found to contain but a single species; they will be, in other words, pure cultures. It is evident that such a method must be cumbersome and poorly adapted to laboratory work for most cases. It has been almost wholly superseded by better and more expeditious methods for most isolations, but it is still used for some organisms which grow only in liquid media.

Isolation by Plating. The discovery and introduction of liquefiable solid media, such as gelatin and agar, greatly simplified the securing of pure cultures of very many organisms. Varying amounts of the material from which isolations are to be made are introduced into tubes of suitable nutrient medium containing gelatin or agar. This medium has been first liquefied by heat and then cooled to a temperature of about 42° C. This temperature will keep the medium liquid and is not high enough to injure the microorganisms introduced. The inoculated medium is then poured into a petri dish. The medium is uniformly distributed over the bottom of the dish which is then placed upon a cool surface to cause the medium to solidify quickly. The petri dish is often called a *plate,* and this process is termed *plating* or *plating out*. It is evident that the microorganisms present are separated from each other. The solidification of the medium prevents them from moving about. They are surrounded with suitable nutrients which have been introduced during the preparation of the medium. In the course of a few hours or days each viable organism will grow: the cells will multiply until the mass becomes visible to the unaided eye as a colony (Fig. 12–2). In most instances each colony originates from a single organism and is therefore a pure culture from which transfers may be made directly to suitable media. The simplicity of this method has made it the one most commonly used in the laboratory.

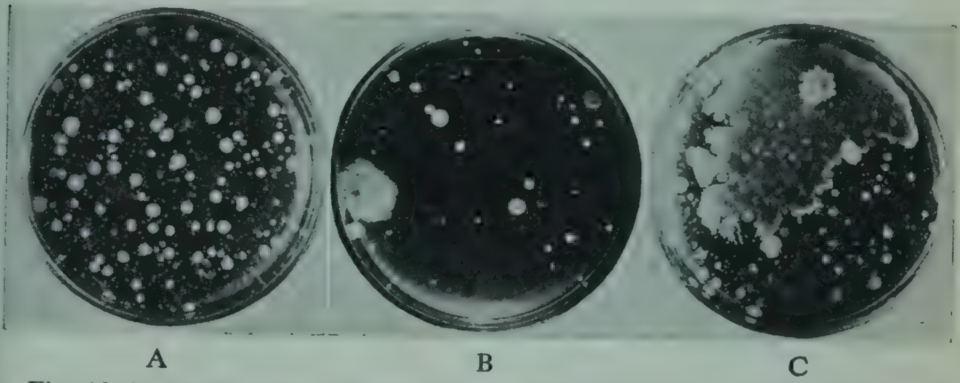

Fig. 12–2. Photographs of plate or petri dish cultures showing the isolated colonies, each presumably representing the progeny of a single cell, and hence a pure culture. Note that the colonies which have grown on the surface of the medium are in general larger than those which are embedded in the medium. Observe that in C one of the colonies has overspread a large portion of the surface, and has overgrown many other colonies. In some cases the spreader apparently has been repelled by certain colonies, and has grown around them.

Isolation of Pure Cultures by Use of Enrichment and Selective Media. An enrichment medium is one which permits the growth of one species or one group of species of microorganisms while inhibiting the growth of others. The term is usually applied to liquid media.

They are particularly useful in the isolation of organisms which are present in small numbers as compared with others. For example, one might wish to isolate cultures of yeasts from soils in which the yeasts are greatly outnumbered by the bacteria present. The problem is to devise a medium in which the yeasts will multiply rapidly with the bacteria increasing slowly or not at all. Yeasts will in general grow well in a medium much more acid than is suitable for most bacteria. Inoculation of such a medium which is sufficiently acid and with the appropriate nutrients will stimulate yeast cell multiplication, hold back the bacterial growth until presently the yeasts predominate, and may be isolated and purified by plating. Similarly, a suitable concentration of bile in certain media inhibits the growth of most organisms other than certain species normally found in the intestines, permitting their isolation and recognition. Use of the enrichment medium technique is very common and has proved most useful.

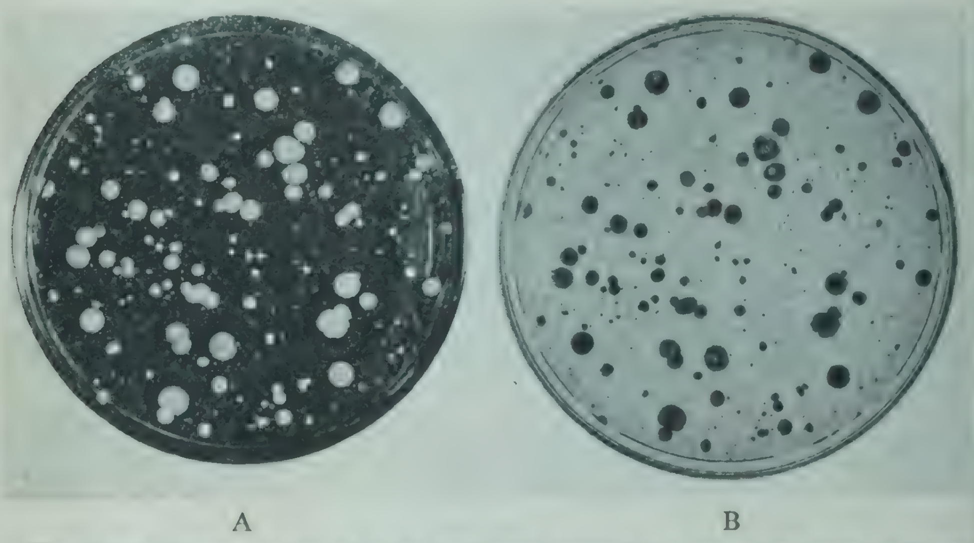

Fig. 12–3. Photographs of plate cultures poured for securing pure cultures from mixed cultures of bacteria. A. The appearance of the colonies with reflected light. B. The same colonies with transmitted light. Transfers from these colonies to a suitable medium would in most cases yield pure cultures.

A solid medium used to produce differentials in growths of microorganisms is sometimes designated as a *selective medium*. Many different substances may be added to such solid plating media as will encourage the development of colonies of certain species as contrasted with others. The reaction of the medium may be used to inhibit certain groups of microorganisms; antibiotics will prevent the growth of some forms and not prevent growth of others.

Isolation by Control of Physical Environment. It may be possible to modify the physical environment so that certain groups of organisms are eliminated, and others are not destroyed. Spore-bearing bacteria may be separated from non-sporulating species by means of heat. Exposure of the mixture of organisms to a suitable temperature for an appropriate time will eliminate the non-spore formers, leaving the spore formers, which may usually be isolated by plating. An exposure to a temperature of 80° C. for thirty minutes has been successfully used in some cases. The spores survive and begin to multiply when the medium is cooled. Purification by other means is necessary if more than one spore-producing species of organism is present.

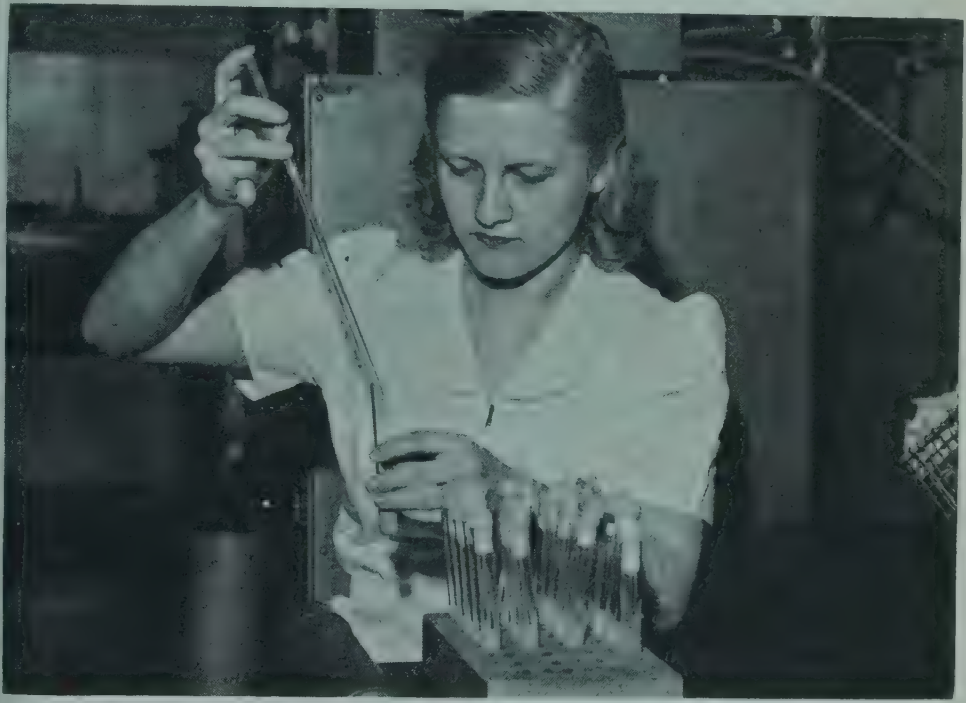

Fig. 12–4. Making dilutions preparatory to pouring of plates for counting, such as shown in Figs. 12–2 and 12–3.

Isolation by Animal Inoculation. Some bacteria capable of producing disease in man or animals do not grow rapidly or readily on culture media, and not at all upon liquefiable media. When these are present in impure cultures, as, for example, the tubercle bacilli in the sputum of a consumptive, it is sometimes necessary to inject the mixture into a susceptible animal, such as a mouse, rabbit, or guinea pig. The body destroys all the bacteria except the one capable of causing disease. The organism desired may then be isolated in pure culture from the blood, or some suitable tissue, such as one of the glands or internal organs.

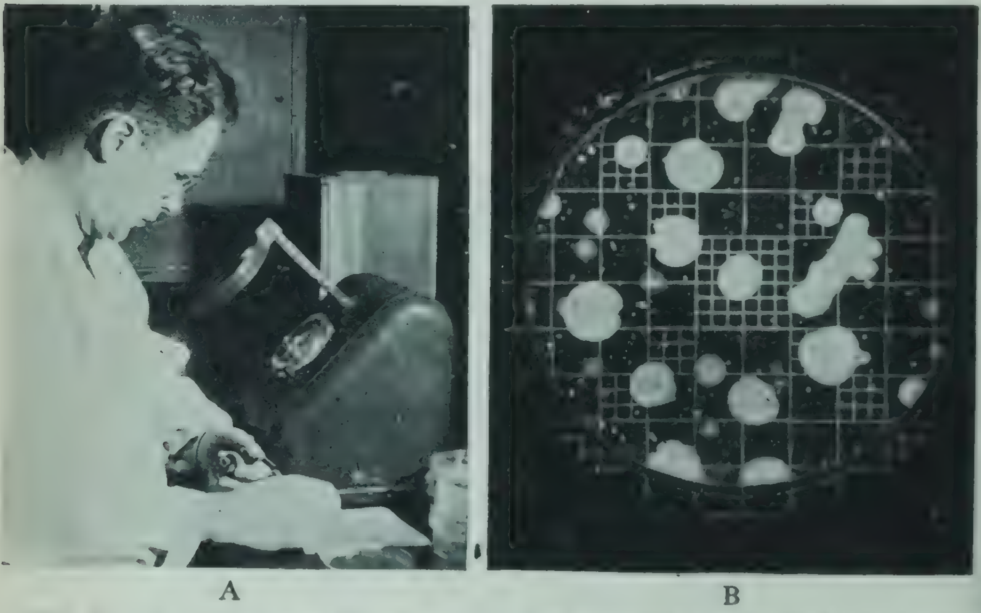

Fig. 12–5. A. Apparatus for counting the numbers of colonies of microorganisms on a plate illuminated from below. Note the large lens, and the counter held in the left hand. B. The plate as seen through the lens. Large surface and small subsurface colonies of bacteria. The ruled lines of the background aid in accurate counting. Each colony is presumed to be made up of the progeny of a single cell. The number of colonies therefore represents the number of viable organisms seeded.

CHAPTER 13

Study of Growth Characters in Pure Cultures

The growth characteristics of bacteria in cultures are often useful in assisting in their differentiation, identification and classification. The same is true with yeasts, and to a less degree with the molds. In the latter, the classification is often based wholly or largely on morphology.

The Society of American Bacteriologists, through a committee, has evolved and adopted a very satisfactory outline chart for recording cultural characteristics. Copies of this chart are kept in most laboratories and should be studied in connection with the contents of this chapter. This chart must be modified in detail in some instances to fit special cases, but is broad enough to cover most types of microorganisms. It will serve as a basis for the following discussion.

CULTURAL CHARACTERS OF BACTERIA

The culture to be studied may be a colony on an agar or gelatin plate, a slant or streak culture on some solid medium, a stab culture in gelatin or agar, or a culture in a liquid medium, such as broth or milk.

Colonies on Agar and Gelatin Plates. The appearance of colonies of the same organism may differ as a result of variations in the composition of the medium. A medium with a moist surface, for example, will give a different type of colony from one whose surface is drier. It is important, therefore, that conditions, both as to composition of the medium and the environment, shall be kept as uniform as possible. It is evident that slight differences in colony form and appearance cannot be made a basis for species differentiation.

Examinations of the colony are made both by means of the unaided eye (macroscopic) and with low magnification (microscopic), using a hand lens, the low power of the microscope or the binocular. Both colonies at the surface and those entirely below the surface (deep colonies) should be examined. The macroscopic examination includes

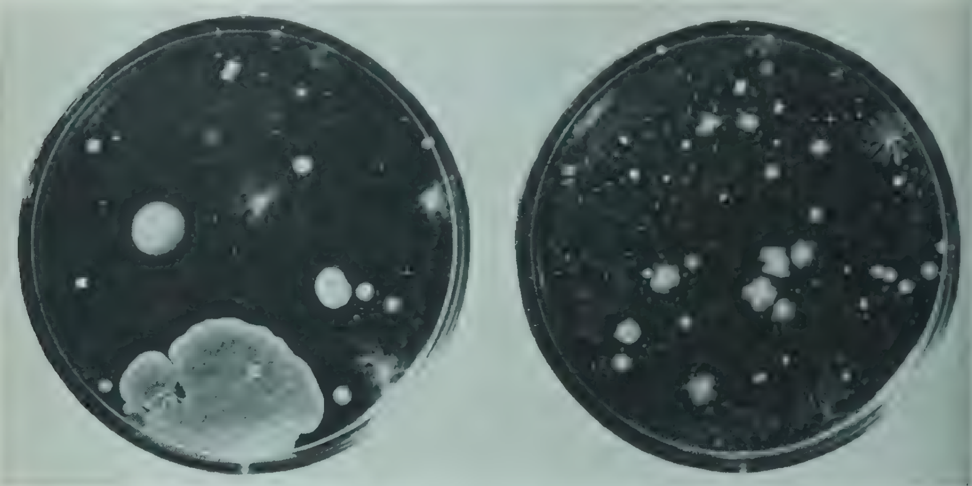

Fig. 13–1. Plate cultures on agar medium showing several types of bacterial colonies. The larger colonies are growing on the surface of the medium. The small colonies, particularly those which are lens-shaped, are subsurface.

Fig. 13–2. Binocular microscope useful in studying the characters of colonies of bacteria, yeasts, and molds. Figures 13–3 to 13–6 illustrate the colony types which may be seen.

a determination of the size, form, surface elevation, margin, topography and consistency.

The diameter of the colony is to be expressed in millimeters. The size is, of course, a somewhat variable quantity, depending upon the age of the culture.

The form of the colony may be described briefly. The following terms are useful, but are by no means the only ones that may be employed for descriptions. A *punctiform* colony is one that is just visible to the naked eye as a minute dot. Colonies are frequently *circular, oval,* or *spindle-shaped* (*fusiform*). An *ameboid* colony is one that is very irregular in shape.

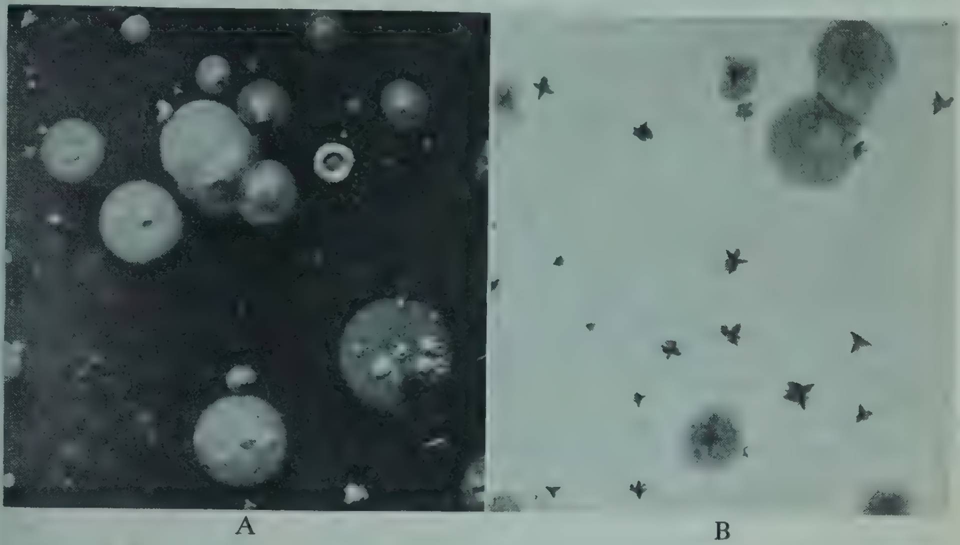

Fig. 13–3. Bacterial colonies on agar plate, somewhat enlarged. A. Reflected light. Note angular deep or subsurface colonies. Secondary colonies are shown in several surface colonies. B. Transmitted light. Note large regular surface and smaller angular subsurface colonies.

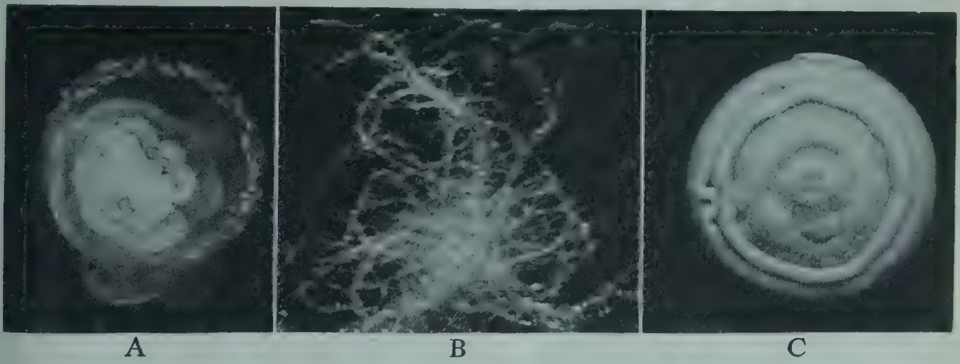

Fig. 13–4. Enlarged surface colonies of bacteria by reflected light. A. Mucoid ridged colony. B. Myceloid colony. C. Rough colony with concentric rings.

The structure of the colony can sometimes be determined by the naked eye; usually a lens is necessary. A *myceloid* colony is one which shows radiating filaments resembling the mycelium of a mold. A *filamentous* colony differs from the preceding in that the threads are irregularly intertwined and not radiating from the center. A *rhizoid* colony is one which shows an irregular, rootlike system of branching.

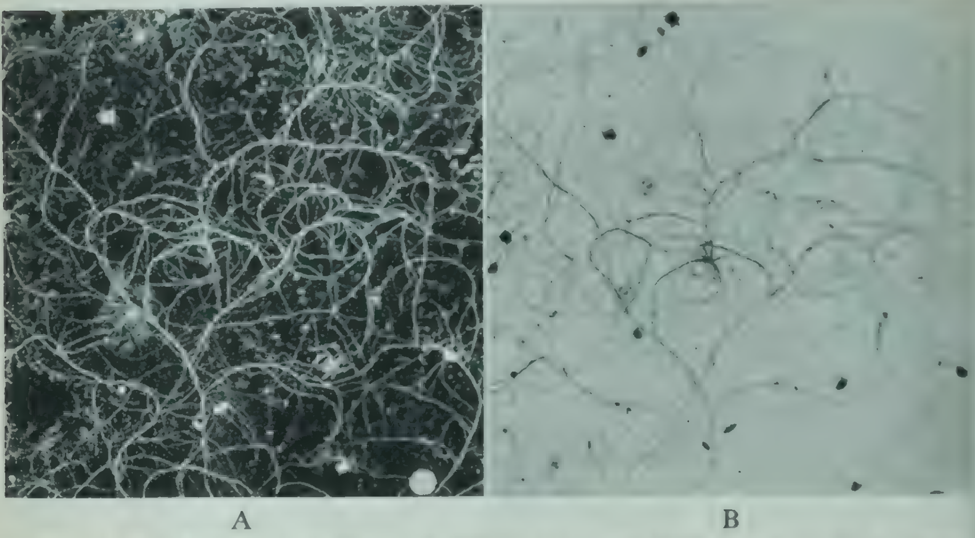

Fig. 13–5. Myceloid bacterial colony. A. By reflected light. B. By transmitted light. Other types of subsurface colonies are also visible.

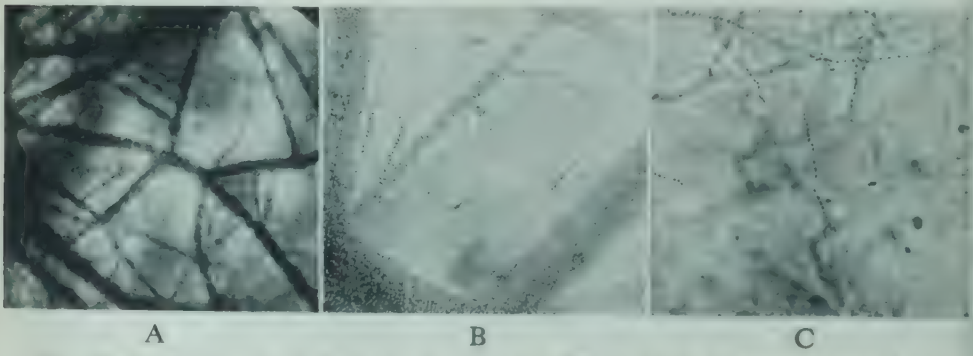

Fig. 13–6. Portions of the same colony in Fig. 13–5 at successively higher magnifications. A. Individual bacteria indistinguishable in the bands of cells. B. At higher magnification the bands and lines are seen to be made up of sporulating bacteria. C. At still higher magnification the chains of spores are quite evident.

The term *surface elevation* indicates the relative thickness of the mass of organisms. A thin, spreading colony is said to be *flat*. If scarcely visible, as a very delicate film over the surface, it is said to be *effuse*. A *raised* colony is one which is uniformly thickened and with a well-defined margin. A *convex* colony is one which is somewhat thicker in the middle than at the edges. A *pulvinate* colony is one which is decidedly convex. A *capitate* colony is one that is hemispherical. A colony having a knob or elevation near its center is said to be *umbonate;* one that has a depression at the center is termed *umbilicate*. If the colony studied is upon gelatin, it may liquefy the medium. The *margin* of a colony, when viewed under the low power of the microscope, may be entire, or without irregularities; *undulate,* or wavy; *lobate,* with rounded lobes; *lacerate,* as if torn or shredded; *fimbriate,* with a fringe; *filamentous,* consisting of filaments like delicate hyphae; or *curled,* like a cluster of hairs.

The *topography* of the surface of a colony may be *smooth, rough, ringed, radiate,* or *striate.*

The *consistency* or *texture* of the colony may be tested by probing with the platinum needle. It may be soft, hard, leathery, butyrous or even brittle.

The *color* and *transparency* of the colony should also be noted.

Rough and Smooth Variants. The tendency of many kinds of bacteria to show variation in morphology leading to variation in colony and growth characteristics has been noted in Chapter 8. The colonies of most kinds of non-spore forming bacteria, particularly those which are pathogenic, tend to be rounded, with margins and surface relatively moist and smooth. These are said to be the *smooth* (S) stage. Many, perhaps most, species of bacteria produce variants having different colony characteristics. The colony is more irregular in shape, the margin is not entire, the surface is rough or uneven. Such colonies are termed *rough* (R) and in pathogenic species tend to be nonvirulent. On the other hand, many of the members of certain genera as *Bacillus* and *Mycobacterium* most commonly produce rough colonies, and the smooth type is the rarer form.

Transfers from smooth colonies tend to remain smooth, as transfers from rough colonies tend to remain rough. The character of the colony must depend on the morphology of the bacterial cells which make up the colony. Frequently there are visible differences: the bacteria in the rough colony may show a tendency to grow in chains, the cell wall apparently may be so modified that ends tend to adhere after cell division and fold, in contrast to the cells of the smooth type which show post-fission slipping movements. The change from *rough* (R) to *smooth* (S) type of colonies, and the converse, is frequently accompanied by other changes. Smooth strains grown in broth, usually cause clouding, the cells tend to distribute themselves rather uniformly; the rough strains have a greater tendency for the cells to remain in masses, forming a film or pellicle at the surface or a sediment at the bottom, the broth remaining relatively clear. Color changes may occur, the rough usually showing less tendency to pigmentation. In motile species of bacteria, the rough forms may be non-motile. Particularly important is the fact that smooth strains are usually the more virulent. There are differences often in serologic characters.

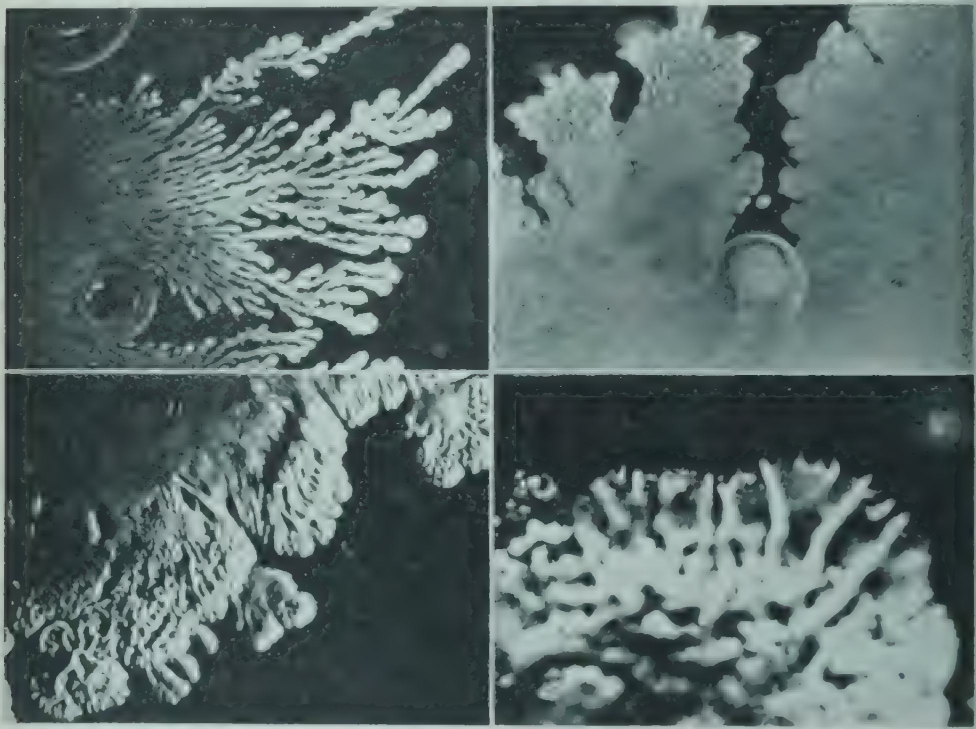

Fig. 13–7. Enlarged margins of rough bacterial colonies by reflected light.

In some species of bacteria, colonies may be found which are intermediate (I) between S and R. The I strains are usually more variable than are either the S or R. Some kinds of bacteria may also produce variant colonies called *mucoid* (M), usually viscid, thicker, and more translucent. The cells produce capsular material or gums which swell by taking up water. Still another type of variant may be shown, the G type, in which the colonies are very small.

An extreme case of the difference between the colony types may be found in cultures of the common soil organism *Bacillus mycoides*. The rough colonies tend to grow rapidly like branching roots over the surface of the medium while the smooth colonies are much more compact and smooth-surfaced. In broth, the R form produces a mold-like filamentous sediment, the S form clouds the medium and develops a uniform sediment.

Rough strains may frequently be obtained from the smooth by growing the organisms in an immune serum or by use of bacteriophage. In some cases the variants show up as papillae or secondary colonies, or they may develop as a variant sector in a colony.

Giant Colonies. In some cases colony characteristics may be accentuated by inoculation of a solidified agar plate with the organism to be studied, so that isolated colonies may develop and grow without interference from other colonies. These colonies may grow relatively large and show many characters not observed in colonies having more limited growth. Such giant colonies are particularly useful in the differentiation of the yeasts.

Slant or Streak Cultures on Solid Media. A slant, slope, or streak culture of an organism is prepared by drawing an inoculated needle in a straight line over the surface of a medium. This medium may be

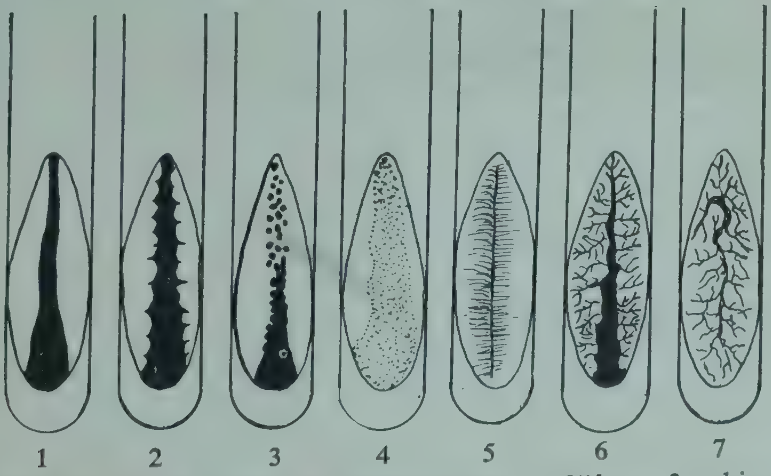

Fig. 13–8. Forms of growth on streak cultures. 1, filiform; 2, echinulate; 3, beaded; 4, effuse; 5, plumose; 6, arborescent; 7, rhizoid.

one rendered solid with agar or gelatin; it may be coagulated blood serum or egg; or the cut surface of a potato, starch paste, etc. It is customary to note in these cultures the amount and form of growth, its elevation, luster, topography, color, odor, consistency, and any changes produced in the medium.

The *amount of growth* may be described as *invisible, scanty, moderate,* or *abundant*. These terms are only relative and some experience is necessary for their differentiation.

The *form of growth* may be *filiform,* extending but little to either side of the line of inoculation; *echinulate,* somewhat wavy or roughened on the margins; *beaded,* the organism growing as more or less minute separate colonies; *effuse* or *spreading,* as a thin indefinite layer over a large portion of the surface; *plumose,* or feathery; *arborescent,* with outgrowths at the side like the branches of a fir tree; or *rhizoid,* with rootlike branches (Fig. 13–8).

The terms describing *elevation of growth* are much the same as those for colonies; those most commonly used are *flat, effuse, raised,* and *convex*.

The *luster* of the culture is determined by the use of reflected light. It may be *glistening, dull,* or *cretaceous* (chalky).

The *topography* of the surface may be *smooth, contoured* (with an irregular and smoothly wavy or undulating surface), *rugose* (wrinkled), or *verrucose* (covered with warts).

The *optical characteristics* are determined by the use of transmitted light. The culture may not transmit light (*opaque*) or it may be *translucent* or *transparent*.

The *chromogenesis* or color production of an organism should be noted. The pigment may remain wholly within the mass of growth, or it may diffuse through the medium. The solubility of the pigment may also be determined in water, ether, alcohol, and chloroform.

The odor may be recorded as *absent, slight,* or *decided*. Possible resemblance to other odors should be noted.

The *consistency* of the culture is most readily determined by touching with a sterile platinum wire. It may be *slimy, butyrous* (resembling butter), *viscid, waxy, membranous* (difficult to detach from the medium, usually the entire growth coming off together), or *brittle*.

The *changes in the medium* may consist of liquefaction of gelatin, blood serum, and similar media, or of a change in color. If the latter occurs, the exact change should be recorded.

Stab or Stick Cultures in Solid Media. These are prepared in media solidified by agar or gelatin, by inserting a straight inoculated platinum needle for some distance. The growth on the surface of the medium may be described in the same manner as for colonies. The principal points to note in these cultures are: growth along the line of puncture and changes occurring in the medium (Fig. 13–9).

The *growth along the line of puncture* may be distributed *uniformly;* it may be *best near the top, best near the bottom,* or occasionally *best at some intermediate point.* It may be *filiform* or uniform; *beaded,* consisting of more or less distinctly separated colonies; *papillate,* covered with small papillae; *villous,* covered with straight hairlike projections; *plumose,* like a feather; or *arborescent,* like a tree with a main trunk and branches.

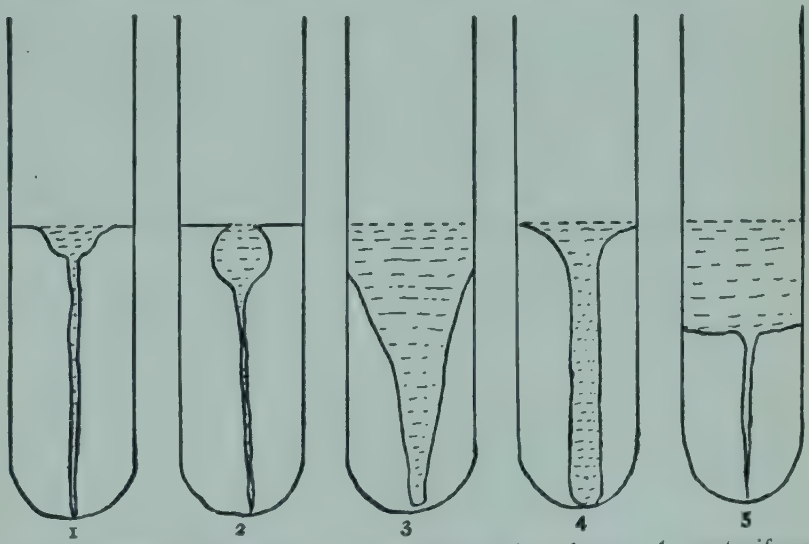

Fig. 13–9. Types of liquefaction in gelatin stab cultures. 1, crateriform; 2, napiform; 3, infundibuliform; 4, saccate; 5, stratiform.

The principal *change in medium* to be noted is liquefaction. The liquefied portion may be *crateriform,* or saucer-shaped; *napiform,* like a turnip; *infundibuliform,* funnel-shaped; *saccate,* sack-shaped; or *stratiform,* as a horizontal stratum. Changes in the color of the medium should also be noted.

Cultures in Liquid Media. These may be made in some transparent medium as broth, or in one of the synthetic media, or milk. In the clear media it is necessary to observe the surface growth, clouding, sediment, and odor. In milk (usually litmus milk) the coagulation, digestion of casein, reaction, and consistency should be noted.

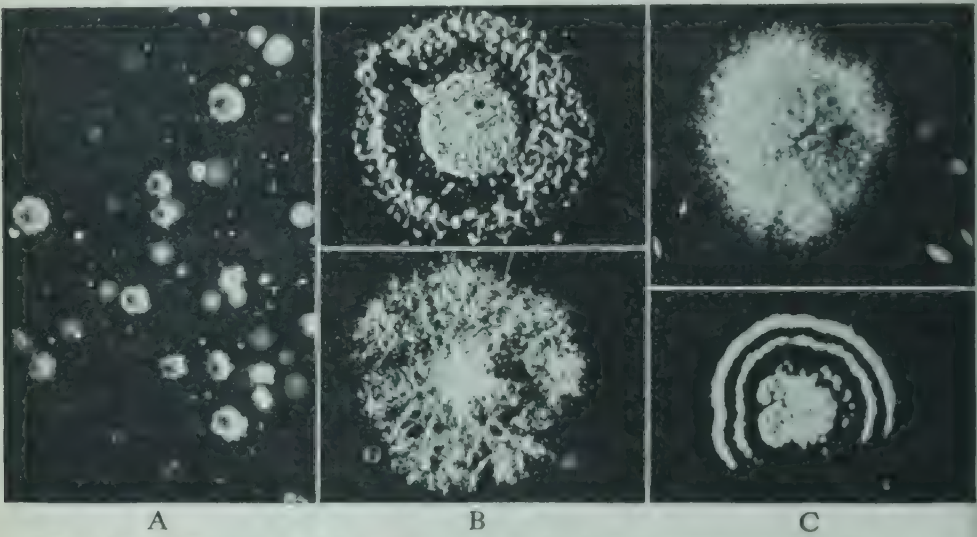

Fig. 13–10. Colonies of true bacteria and of actinomycetes. A. Colonies of *Streptomyces* (powdery) and of bacteria (smooth). B and C. Higher magnifications of colonies of various species of *Streptomyces.*

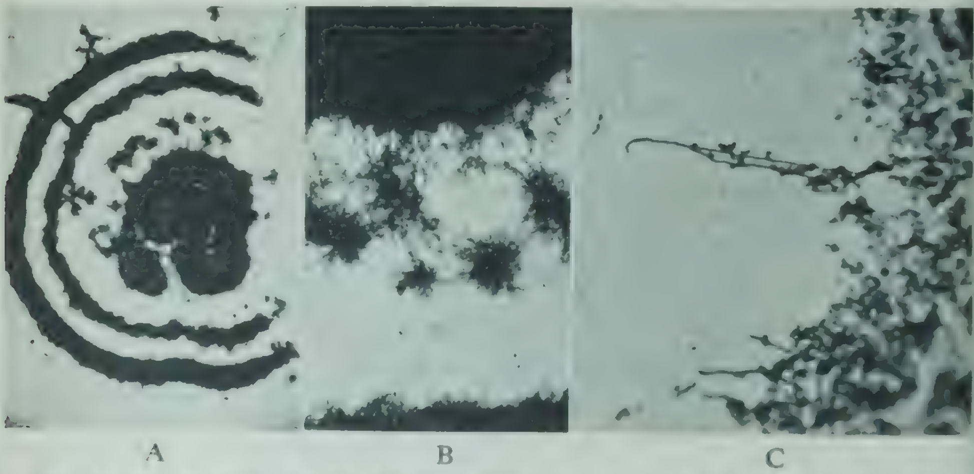

Fig. 13–11. Colony of an actinomycete, at successively higher magnifications. A. Colony somewhat enlarged, showing ring formation and mycelial clumps. B. At higher magnification, showing the mycelium. C. Still higher magnification, showing individual hyphal threads and spores.

Some of these latter changes will be discussed in greater detail under the heading of physiological characters.

Surface growth in broth may occur as a ring where the surface of the medium meets the walls, or as a more or less definite membrane or *pellicle*. It may remain persistently at the surface or it may be easily shaken down to the bottom. It may be *friable, firm, membranous,* or even *leathery*. With many organisms no surface growth appears.

The clouding of broth may be *absent, slight, moderate,* or *strong.*

The *sediment* may be *absent, scanty,* or *abundant* in amount, and in consistency *compact, flocculent, granular,* or *viscid.*

Milk may be curdled promptly, after the lapse of considerable time, or not at all; and the curd if formed may or may not shrink with expulsion of the whey, and it may or may not be digested. The curd may be produced as a result of the development of acid or of a rennet-like enzyme. Most organisms that digest the casein actively produce coagulation by the second method; the acid-forming bacteria in most cases do not digest the casein. The reaction may remain unchanged, or it may become acid or alkaline, or first acid and then alkaline. The consistency of the milk may remain unchanged, or it may become slimy or viscid.

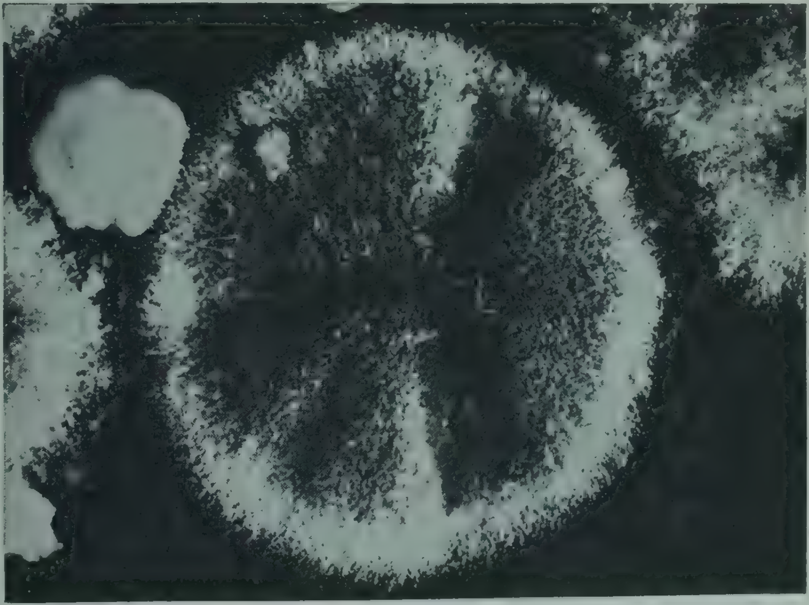

Fig. 13–12. Mold colony showing light and dark sectors.

CULTURAL CHARACTERS OF YEASTS AND MOLDS

The coloring and growth characteristics of yeasts are studied as are the bacteria. The same descriptive terms may be used. The types of media best adapted for their growth, however, are not always the same as those used for bacteria. Sugar broths, synthetic media, wort, and must, with or without the addition of agar or gelatin, are most commonly used for their cultivation. The occurrence of film formation on certain liquid media may be particularly significant. Giant colonies on beerwort or a sugar agar are often useful in differentiation.

Media adapted for mold growth are of many types, including not only the common laboratory media, particularly those useful for yeasts, but also many substances such as breads, fruits, grains, and other foods and foodstuffs.

The study of a mold culture should include a determination of the *size of the colony* and of its *rapidity of growth*. The *general appearance,* whether velvety, smooth, cobwebby, cottony, etc., should be noted. An attempt should be made to see the spore-bearing organs with the unaided eye. The *color* of the mold and each of the separate parts should be recorded. Any *changes* in the *color* or *consistency* of the medium on which it is growing should be carefully noted.

CHAPTER 14

Methods of Observation of Certain Physiological Characters

Only the *methods* that are used in the detection of chemical and physical changes in the environment will be discussed in this chapter. A more extended discussion of the changes produced by bacteria, yeasts, and molds will be taken up under the heading of physiology.

Acid Production. Many bacteria when grown in nutrient solutions, particularly those containing carbohydrates, produce acids and bring about thereby marked changes in *hydrogen ion concentration.*

Determination of Hydrogen Ion Concentration. In Chapter 11 were discussed methods of adjusting the reaction of a medium. It was there noted that the hydrogen ion concentration (or activity) depends upon several factors. In culture media in which microorganisms are grown, the factors most significant in producing change in the pH are: first, the *amount* of acid produced; second, the *kind* of acid produced; third, the *amount of buffer* present in the medium; fourth, the *amount of base* produced. Alkalinity may be developed either by the production of ammonia, or by the transformation of the salt of a strong acid to the salt of a relatively weak or little dissociated acid. For example, certain bacteria may transform sodium citrate into sodium carbonate; solutions of the latter are much more basic than the former.

Several methods have been developed for measuring the hydrogen ion concentration of a solution and for detecting changes. One of these is electrometric in which use is made of the hydrogen electrode. This involves a somewhat complicated setup for a measurement of the increased electric potential of the solution due to the production of hydrogen ions. When accurate records are to be secured, this is the preferred technique. For much laboratory work, simpler though perhaps slightly less accurate methods are commonly employed. One of these, the colorimetric method, is simple and ordinarily used. A suitable *indicator* is added to a portion of the solution to be tested, which may be diluted somewhat with distilled water. The color of the solution is then compared with the color produced by similar addition of indicator to standard solutions whose hydrogen ion concentrations are known and the colors matched. Indicators of many kinds have been described. Extracts of many plants and animals have been used, as litmus from a lichen, cochineal from an insect, red cabbage extract and pigments from various flowers. Most of them are poorly adapted to the needs of the bacteriologist. The indicators most used in the bacteriological laboratory for this purpose are those developed by Clark and Lubs. In the following table the name of each of these indicators is given, followed by the color in its acid range, next the color in its alkaline range, and finally the range of pH values through which it changes color.

COLOR CHANGE OF CLARK AND LUBS INDICATORS

Indicators	*Full Acid Color*	*Full Alkaline Color*	*Sensitive Range**
Thymol blue (Acid range)	Red	Yellow	1.2–3.8
Brom phenol blue	Yellow	Blue	3.0–4.6
Methyl red	Red	Yellow	4.4–6.0
Brom cresol purple	Yellow	Purple	5.2–6.8
Brom thymol blue	Yellow	Blue	6.0–7.6
Phenol red	Yellow	Red	6.8–8.4
Cresol red	Yellow	Red	7.2–8.8
Thymol blue (Alkaline range)	Yellow	Blue	8.0–9.6
Phenolphthalein	Colorless	Red	8.0–9.6
Cresolphthalein	Colorless	Red	8.2–9.8

* The indicator changes from the acid color to the alkaline color between the following pH values

It is apparent that an approximation of the pH may be secured by using different indicators in portions of the liquid being studied and determining which gives color in the acid range, and which an alkaline color. For example, if it is found that phenol red when added to the solution gives a yellow color, the medium is acid and must be below the pH value of 6.8. If methyl red, on the other hand, gives a yellow color, it must have a pH value above 6. In such case the approximate pH value can be determined then by use of brom thymol blue, comparing the intensity of the color change from yellow to blue with standard solutions whose pH values are known.

Differences in the hydrogen ion concentration produced by different species of bacteria are frequently of use in their identification and classification. This is the basis of the *methyl red test* which will be discussed later as a method useful in the differentiation of certain bacteria of importance in determining the potability of water.

For discussion of the methods of preparing standard solutions and colors for accurate determinations of pH a suitable laboratory manual should be consulted.

Quantitative Determination of Acid Production. What has been stated above concerning determination of hydrogen ion concentration will indicate the method of determining whether or not acid has actually been produced by an organism. It should be noted, however, that the determination of hydrogen ion concentration is not a determination of the total amount of acid present or produced. Measurement of hydrogen ion activity is a measurement of an *intensity* factor, it is not a measurement of a *capacity* factor. For this latter a comparative titration is made. It is customary to titrate to a definite tint a sample of the sterile medium retained as a check, using phenolphthalein or some other suitable indicator. For this purpose place five milliliters of the sterile material in a porcelain evaporating dish, add forty-five milliliters of distilled water, and a drop or two of alcoholic solution of phenolphthalein, then from a burette add twentieth normal (0.05N) alkali until a definite pink color has been established, noting the amount of alkali required. A similar titration is made using the medium in which the organism under study has been grown. The difference between the amount of alkali required to bring the two samples to the same tint of red is a direct measure of the amount of acid produced. This may be calculated in grams per liter if the kind of acid formed is known.

This method of acid determination is not absolutely accurate inasmuch as there may be basic substances developed simultaneously with the formation of acid from the substrate.

In some cases it is desirable to determine the kind of acid which has been produced. Simple chemical tests for the various organic acids have not in most instances been developed. Something of their nature, however, may be determined by separating them into volatile and non-volatile. If a solution containing an acid is acidified with sulfuric acid and distilled, certain acids, particularly acetic, propionic, and butyric will pass over, while other acids, particularly lactic, will not. It is then possible by titration of the distillate to determine the relative proportion of the two groups of acids.

Measurement of Oxidation-Reduction Potential. An environmental factor which is always important in cultures of microorganisms and one which must sometimes be measured, is the tendency of the medium to induce oxidation on the one hand or reduction on the other. It is necessary to measure and to understand something of the significance of oxidation-reduction potential.

First, we should review the meaning of the terms oxidation and reduction. Originally, oxidation as applied to a chemical change meant the addition of oxygen to a molecule. Formaldehyde may be oxidized to formic acid by the addition of oxygen.

$$2HCHO + O_2 \rightarrow 2HCOOH$$

Similarly, reduction was conceived as the removal of oxygen. Then it was recognized that oxidation may be the result of the removal of hydrogen. Methanol may be oxidized thus to formaldehyde.

$$CH_3OH \rightarrow HCHO + H_2$$

Reduction conversely may be the addition of hydrogen. Then still a third type of oxidation was discovered, involving a change of valence of ions. Iron as ferrous chloride may be oxidized to iron as ferric chloride. In this case no oxygen is added and no hydrogen removed.

$$FeCl_2 + Cl \rightarrow FeCl_3$$

The chemist has shown that the essential change is that ferrous chloride in solution ionizes to ferrous ion and chlorine ions

$$FeCl_2 \xrightarrow{H_2O} Fe^{++} + 2Cl^-$$

in which (+) indicates a single positive charge of electricity and (−) a single negative charge or *electron*. Then

$$Fe^{++} + 2Cl^- \overset{Cl}{\rightleftarrows} Fe^{+++} + 3Cl^-$$

The iron gives up a negative charge or electron to the chlorine and in consequence has three positive charges. This reaction may go in either direction. When it goes to the right, when iron is losing electrons, oxidation is said to be taking place. When the reverse change occurs and the iron takes up an electron, it is thereby reduced. This may be written (e = electron).

$$Fe^{++} \underset{\text{Reduction}}{\overset{\text{Oxidation}}{\rightleftarrows}} Fe^{+++} + e$$

Does this mean that there are three kinds of oxidations and reductions, one kind involving oxygen, one involving hydrogen, and one involving electrons? It has been shown that not only in the oxidative change in valence but also in all other oxidations, the essential change is loss of electrons. Indeed, oxidation may be defined as a chemical process involving the loss of electrons, and reductions as one in which there is a gain in electrons.

However, an electron which leaves an atom which is thereby oxidized must go to some other atom that is simultaneously reduced. In other words, whenever oxidation occurs, there is a simultaneous reduction.

In the biological oxidations and reductions that occur in the cell and in the media in which microorganisms grow, usually oxidation is accompanied by loss of hydrogen and reduction by its acquisition. The work of Wieland and of Kluyver has shown this concept to be most helpful in understanding the chemical changes produced by microorganisms. Compounds which readily are oxidized by loss of hydrogen and electrons are termed *hydrogen donors,* and those which are readily reduced by taking up hydrogen and the accompanying electrons are called *hydrogen acceptors*.

In any medium in which microorganisms are grown there will be many substances which tend to be oxidized and many others which tend to be reduced. Some tend to give up electrons, others to take them up. The intensity of this tendency to oxidize or reduce is evaluated by determination of the *oxidation-reduction potential*. How may it be measured?

The basic determination of the oxidation-reduction system is made by comparison with that of a standard by means of a potentiometer. For careful or accurate determinations in the bacteriological laboratory the electrometric method is used.[1]

However, just as intensity of hydrogen activities (pH) can be approximated by indicators properly chosen, so here the E_h may be approximated by use of indicators which show change in color over a definite E_h range. The most useful are those which become decolorized when reduced.

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[1] The unit of measurement is the *volt*. The oxidation-reduction potential (E_h) is the difference in potential between the solution and that of a normal hydrogen electrode.

Several dyes and pigments are utilized in the laboratory as indicators of changes in oxidation-reduction potentials. The ability of organisms growing under standard conditions to produce decolorization may be of diagnostic significance. Litmus milk, for example, may be completely decolorized by growth of certain species of bacteria. The stability of sewages and wastes may be determined by adding to appropriate dilutions a dye such as methylene blue. If kept in a filled and tightly stoppered container and incubated, the length of time required for the decolorization of the methylene blue is an index to the putrescibility of the sewage and of its need for treatment.

Similarly, methylene blue may be added to milk, which is then incubated; the length of time required for decolorization is an index to the initial contamination of the milk.

Gas Production. Most kinds of living cells (not all) produce the gas carbon dioxide. When formed by cells living in the presence of air, the gas diffuses into the air and is not noted unless special provision is made to collect it. Plant cells which contain chlorophyll (leaf green) when irradiated by light of correct wavelengths produce oxygen when supplied with carbon dioxide.

Microorganisms which can ~~be~~ grow in the absence of air also may produce gases. In some cases the gases formed, commonly carbon dioxide and hydrogen, less commonly nitrogen or methane, are produced in sufficient quantities to be readily recognized. Ability to produce gas constitutes a useful criterion in the identification of many organisms.

Yeasts and some bacteria produce only carbon dioxide from carbohydrates, other bacteria produce both carbon dioxide and hydrogen. A qualitative test of the ability of an organism to form gas may be made by inoculating a tube of liquid agar or gelatin containing a suitable carbohydrate. If gas is evolved, the medium will be broken by numerous bubbles. For a study of the ability of an organism to produce gas from various carbohydrates large and small test tubes may be used. A suitable quantity of the test medium is placed in a large tube, a smaller tube is inverted and dropped in. During sterilization the air in the small tube is replaced with water vapor which condenses as cooling takes place, and the medium rises to fill the inner tube. When inoculated, the gas formed collects in part in the inner tube. For a quantitative determination, it is customary to use a fermentation tube. The closed arm of this tube is entirely filled and the open arm partially filled with a liquid medium containing the carbohydrate under investigation. After sterilization, the organism may be inoculated into the open arm, and any gas produced in the closed arm will collect there. The amount of gas which forms may be determed by the use of a Frost gasometer (Fig. 14–1). An approximate determination of the composition of the gas produced may be made by filling the open arm with normal sodium hydroxide and covering the opening with a bit of rubber tissue held in place by the thumb, using care that no air bubbles are included. The gas which has collected in the closed arm may now be introduced into the open arm and shaken vigorously in contact with the alkaline solution. After a few minutes, it may be returned to the closed arm and the thumb removed. Any *carbon dioxide* present in the gas will be absorbed by this procedure, and the liquid will rise in the closed arm to replace it. The gas remaining, if any, is usually *hydrogen*. Its presence may be demonstrated by allowing it to pass into the open arm and bringing it near a flame, when a slight explosion will occur. The *ratio* of carbon dioxide to hydrogen in the gas produced by bacteria is sometimes used as one of the characters differentiating species. The carbohydrates most commonly employed for this purpose are dextrose, lac-

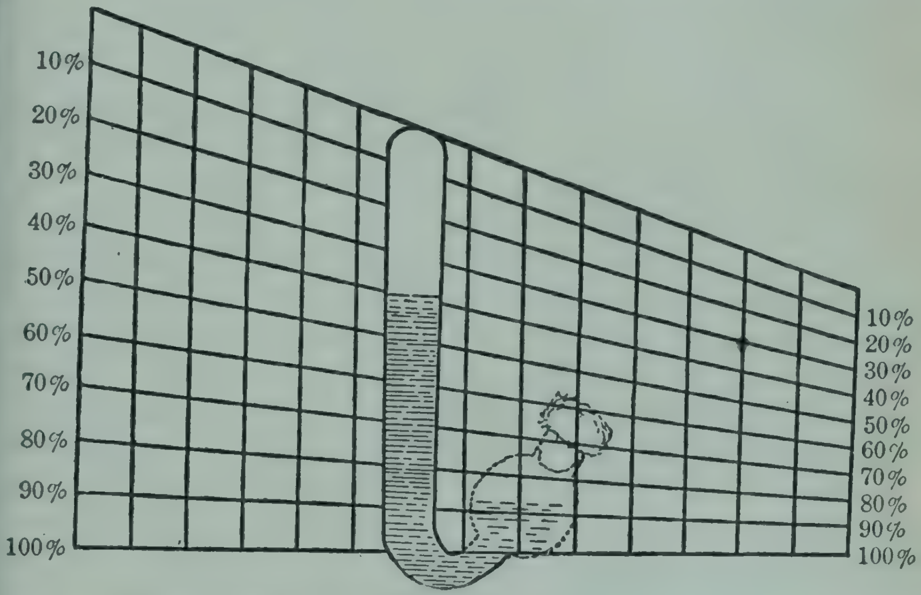


Fig. 14–1. Gasometer (Frost's). The amount of gas produced in the fermentation tube is read off directly from the gasometer.

tose, and saccharose. For more detailed study some twelve or fifteen other carbohydrates and higher alcohols are used.

Alcohol Production. Ethyl alcohol is produced by a few bacteria and molds, and by most yeasts. Small quantities of amyl, butyl, and propyl alcohol are also formed by a few microorganisms. Alcoholic fermentation occurs in solutions containing suitable carbohydrates and is accompanied by the evolution of carbon dioxide. A simple qualitative test for alcohol in a solution may be carried out by adding to a few cubic centimeters of the liquid in a test tube a small crystal of iodine and several cubic centimeters of sodium hydroxide solution. This should be heated over the flame of the bunsen burner, when, if alcohol is present, the odor of iodoform may be readily detected. Quantitative estimations are made by distillation, followed by determination of specific gravity of the distillate.

Aldehyde Detection. Microorganisms which actively utilize carbohydrates usually form detectable amounts of aldehydes as intermediate products in their metabolism. The proof that carbohydrate is utilized by an organism is frequently accomplished by detection of the aldehyde through the addition of a sulfite which blocks its usual prompt utilization by the organism. The red dye basic fuchsin is added to the medium during its preparation, followed by a solution of sodium sulfite which unites with the fuchsin to form a colorless compound. If aldehyde is formed in the medium by the growth of an organism, it unites with the sulfite present and frees the dye fuchsin and the red color is restored. This is the principle made use of in the so-called Endo agar medium. Certain bacteria when growing upon this medium form red colonies because the fuchsin is freed owing to the development of aldehyde and acid. Other species of bacteria grown upon this medium do not change the color.

Acetyl Methyl Carbinol (Acetoin). This compound is produced by certain bacteria growing in the presence of carbohydrates. It is recognized by the addition to the medium of strong alkali such as sodium hydroxide or potassium hydroxide. When allowed to stand for a few hours an eosin pink or red color will develop, particularly near the surface, provided both acetoin and peptone are present. This is frequently called the Voges-Proskauer (V-P) reaction, after the men who first noted it.

Reduction of Nitrates to Nitrites. Certain microorganisms, particularly bacteria and some yeasts, when grown in a nutrient solution in the presence of nitrates, reduce the nitrates to nitrites. This chemical change may sometimes aid in the differentiation of species. The change is evidently a reduction, the organism probably making use of the nitrate as a hydrogen acceptor. For detection of nitrate reduction a broth is prepared containing 0.1 per cent peptone and 0.02 per cent potassium nitrate. The inoculated tube is allowed to stand for four days, and is then tested for nitrites by use of solutions *a* and *b*.

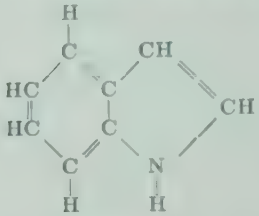
a.	5*N* acetic acid	1000 ml.
	Sulfanilic acid	8 g.
b.	5*N* acetic acid	1000 ml.
	1-naphthylamine	5 g.

Two cubic centimeters of each solution are added to the tube of medium to be tested; if nitrite is present, a red or rose color will develop. An uninoculated tube should always be tested at the same time as a check or control.

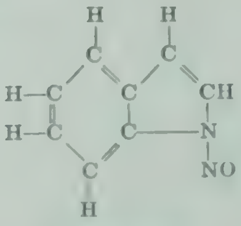
Reduction of Sulfates. Some organisms reduce sulfates to sulfides. This transformation may be detected by the addition of an iron salt, as the chloride, to the solution, when the black iron sulfide will be precipitated. Or the medium may be heated and lead acetate paper exposed to the vapor; hydrogen sulfide will cause this to blacken. Lead acetate incorporated into a medium may be used to detect the formation of sulfides through the precipitation of the black lead sulfide.

Production of Ammonia. Many organisms produce ammonia when grown in a medium rich in proteins or peptones. A qualitative test for ammonia may be carried out by heating the medium in which the organism has been growing and exposing a bit of filter paper dipped in Nessler's solution to the vapor. It will be turned red, brown, or black if ammonia is present.

Indole Production. Indole is formed by many species of bacteria when grown in a medium rich in protein or peptone containing tryptophan and little or no sugar. It is customary to test for indole production by growing in peptone water or tryptophan broth. The tube should be incubated for several days before being tested. The qualitative test for indole is carried out by the addition of a cubic centimeter of 0.1 per cent solution of sodium nitrite and a few drops of sulfuric acid. The nitrite is decomposed by the action of the sulfuric acid and nitrous acid is freed. This unites with the indole to form a red compound known as nitroso-indole. Numerous other color reactions for the recognition of indole have been developed, some of them much more delicate than the foregoing, among them the Ehrlich reaction.[2] The production of indole furnishes a ready method of differentiating certain species of bacteria. The structural formulas of indole and nitroso-indole are as follows:



Indole



Nitroso-indole

Digestion of Starch. Many organisms, when grown in a suitable medium decompose starch. The progress of this digestion may be noted from time to time by removing small amounts of the medium and adding to these a drop of a dilute solution of iodine. The presence of starch is indicated by the blue or purple color which develops. Organisms may be plated in starch agar. After colonies have appeared, the plates may be flooded with a dilute solution of iodine. Uncolored areas will be visible about colonies which digest starch.

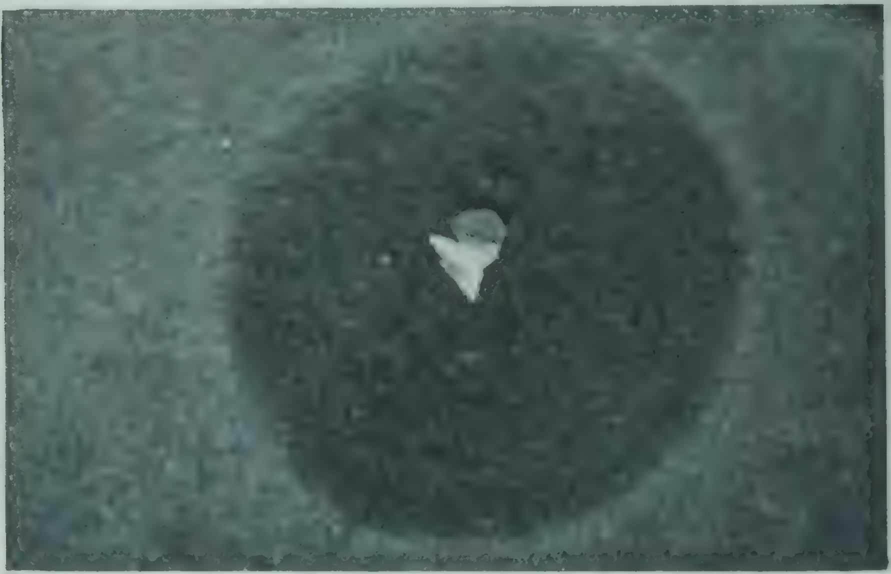


Fig. 14–2. Angular subsurface colony in agar plate, showing clearing of the medium due to diffusion of an enzyme.

Digestion of Gelatin. Microorganisms that digest or liquefy gelatin bring about this change through the agency of an enzyme called

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[2] The Ehrlich reagent is made of 4 g. p-dimethylaminobenzaldehyde dissolved in a mixture of 380 ml. ethanol and 30 ml. concentrated HCl. When added carefully to the liquid culture, the reagent forms a top layer. The presence of indole is evidenced by development of a red color at the surface of the medium.

gelatinase. The ability to liquefy gelatin is an important characteristic in the differentiation of groups of microorganisms, particularly certain of the bacteria. Some bacteria which liquefy gelatin do not break it down into simple compounds; others decompose it with formation of amino acids and ammonia. In other words, some organisms apparently produce gelatinase only, others produce a trypsin-like enzyme as well.

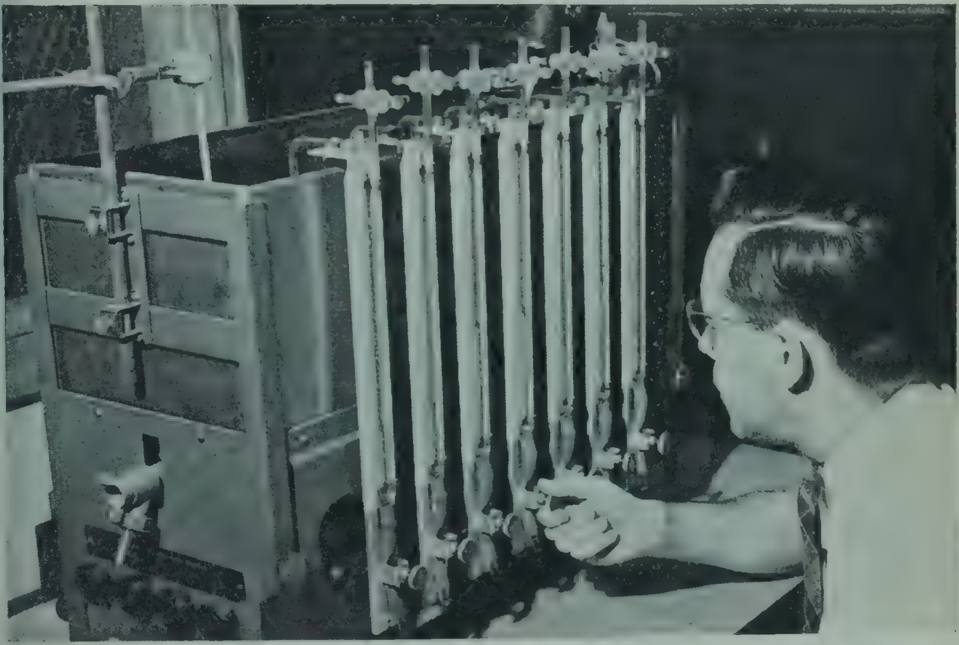

Fig. 14–3. Warburg apparatus for study of cellular physiology. Small glass vessels are attached to the manometers and submerged in the water bath. Changes in amounts of gas in these vessels containing the organisms under study may be read on the manometer scale. Observations can be made on oxygen uptake, carbon dioxide production or assimilation, respiration, etc.

Digestion of Casein. Many organisms when grown in milk first coagulate it and later digest the casein. All milk cultures must be carefully examined to determine whether such digestion of casein takes place.

The digestion of casein may also be conveniently studied by means of casein agar plates. A small amount of casein solution added to agar renders the medium somewhat opaque. Colonies of organisms capable of digesting casein when grown in this medium show a clearing, the medium becoming transparent immediately about the colony. The differentiation may be intensified by flooding the plate with a dilute solution of acid. This will precipitate the casein, the area about the colonies of casein digesters remaining clear.

CHAPTER 15

Methods of Microscopic Examination

Two topics should be considered, the special uses of the several types of microscopes for the observation of microorganisms in the microbiological laboratory, and the preparations of microorganisms for microscopic examination.

MICROSCOPES AND SPECIAL USES

The microscopes that can be of special service in the bacteriological laboratory are the low power "dissecting" binocular, the compound microscope with the oil immersion objective and the dark-field illuminator, the quartz lens system for compound microscope to be used for photographing by ultraviolet light and for observation of fluorescence, the phase microscope and the electron microscope.

The Binocular "Dissecting" Microscope. This is particularly valuable for study of the colony characteristics of bacteria, yeasts, and molds. The use of a good microscope lamp will enable one to study cultures both by reflected and by transmitted light.

The Compound Microscope. The lower power dry objectives are frequently used for examination of colonies and for study of the larger microorganisms such as the molds and some of the protozoa.

For study of the morphology of the bacteria, yeasts, and many of the molds and protozoa, it is usually necessary to use $^{1}/_{12}$ inch or 1.8 to 2 mm. homogeneous oil immersion objectives. A drop of immersion oil is placed on the stained preparation or on the cover glass over the object to be examined, and the objective is lowered until it is immersed in the oil. This oil must always be used with a lens of this type. The following explanation of the reason for using the oil may be given. Consult the diagram in Fig. 15–1. Let *C* represent the glass microscopic slide, *L* the lens at the end of the objective having an opening between *F′* and *F*, and *H* a drop of oil lying between the objective and slide. This oil should have the same refractive index as glass. The rays of light are focused by means of the mirror and Abbé condenser, being concentrated at the point *B*. It is evident that any ray of light, such as *BN*, which strikes the slide at right angles, passes straight through and enters the objective. Another ray, such as *AB*, striking at a considerable angle, is refracted on entering the glass in the direction *BD*, and on re-entering the air passes in the direction *DE*. Such a ray does not enter the lens. Consider, on the other hand, the ray *A′B*, which is refracted as *BD′*, and enters the oil *H*. It is not refracted here and passes in the direction *D′F*. This ray can enter the lens, resulting in a more brilliantly illuminated field and a clearer definition than would be the case in the absence of the immersion oil.

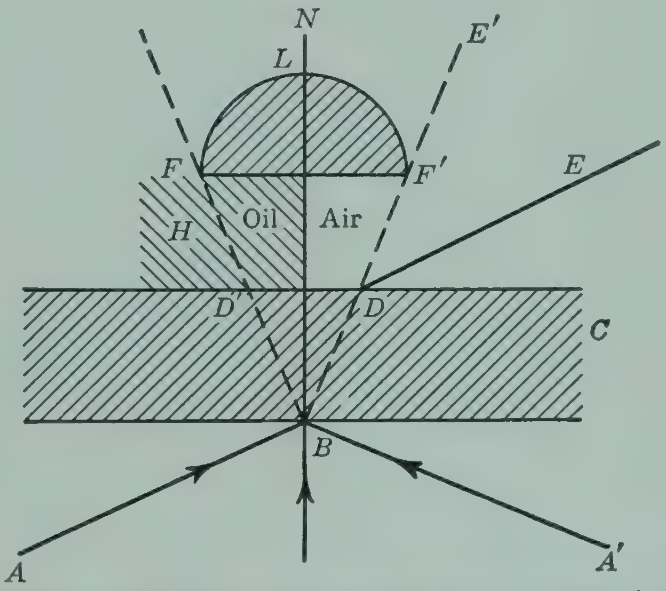

Fig. 15–1. Oil immersion lens and object glass in diagrammatic longitudinal section.

Dark-Field Illuminator. In the usual observation of microorganisms under the microscope, they are seen by means of transmitted light. Differences in refraction of different parts of the organism and differences in color and transparency make possible the differentiation of the parts of the cell. It is possible also to examine microorganisms by light reflected from the cells and their parts or by a combination of reflected and refracted light. Various methods have been devised for insuring the observation of microorganisms as brightly illuminated objects in a dark field. Even objects too small to be seen by transmitted light are visible through the light emitted. The device most commonly used in the bacteriological laboratory for this type of study is the *dark-field condenser* which is substituted for the usual substage condenser. In the dark-field condenser most of the central beam of light from the substage is eliminated, and the marginal rays are reflected and refracted so that they enter the field at such an angle that they will not enter the lens. If one looks through the microscope when there are no objects in focus, as at the surface of a clean, smooth glass microscope slide, the field appears uniformly dark. If, however, there are objects in the field which can reflect (or refract) light, some rays will have their direction so changed as to enter the lens, the object thereby becoming visible. One may even observe the flashes of light from ultramicroscopic objects, he may see the light without seeing the object itself. The flagella of bacteria which usually are quite invisible in bright-field examination in unstained living bacteria may be observed by use of the dark field technique.

The Phase Microscope. Still another device developed in recent years has proved useful in observation of details in microorganisms, particularly in living cells. The explanation of precisely what is meant by the term phase microscope is somewhat complicated; knowledge of the basic theory is *not* a deterrent to satisfactory use. Briefly, in the phase microscope the light is so controlled in its passage through the microscope that changes in phase in the light which passes through various parts of the cells are converted into differences in light intensities which are readily observed by the eye.

The Electron Microscope. The greater magnification and the better resolution to be secured in the study of microorganisms by the use of the electron microscope enables the investigator to observe details quite beyond the resolution capacities of the light microscope. The electron microscope is a relatively large instrument, it is expensive from the standpoint of first cost and of maintenance, and it must be operated for best results by one who has achieved a high degree of technical proficiency. There are also certain difficulties inherent in its use. The objects such as microorganisms that are to be examined must be dry and mounted on a thin membrane of a nature that will not interfere with the penetration of the electrons. This membrane is usually supported on a metal grid for strength. It is necessary that the working parts of the microscope be operated in a relatively high vacuum, for the electrons are absorbed by the atmosphere or by other gases. Magnets are used to bend and focus the electron beams in a manner quite analogous to the use of the glass lens for refracting and focusing light rays in the light microscope. The image of the object under examination is focused upon a glass screen coated with a film which fluoresces when exposed to the beam of electrons. The object under examination casts a shadow on the screen, this shadow varying in depth with the ability of the object to cut off the passage of the electron beam.

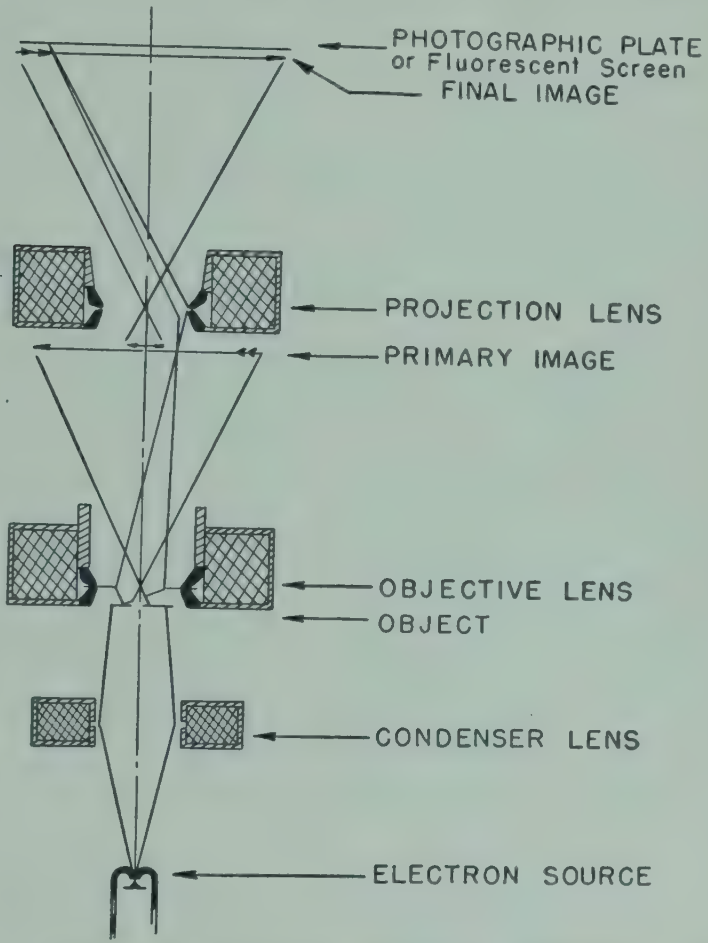

Fig. 15–2. Diagram of the optics of the electron microscope. The "lenses" are electromagnets which bend the paths of the electrons as indicated in a manner analogous to the bending of the rays of light by the glass lenses of the light microscope.

Figure 15–2 illustrates the "optical" principal of the operation of the electron microscope, but gives no hint of the intricacies of the governing and control mechanisms involved.

So-called shadowing of the object under examination has been found frequently to be helpful in getting at general morphology. This is accomplished by exposure of the mounted specimen to a stream of metal vapor, such as the vapor of gold, from one side. The metal tends to coat the side exposed with a thin non-transparent layer; the other side of the particle and the membrane in the shadow are not coated. When an object thus treated is observed in the electron microscope, the surfaces are often brought into striking relief and a much more convincing picture is seen on the fluorescent screen.

The photographic film is also sensitive to the impact of electrons, so it is possible to get satisfactory "electronographs."

Obviously the electron microscope is an instrument of great value in research, but it is not well adapted to the routine work of the student laboratory.

Direct Microscopic Examination of Colonies and Cultures. Cultures growing on agar or gelatin plates may be studied by inverting the plate and examining under the low-power objectives. When practicable, it is better to remove the cover of the petri dish and examine without inversion. The characters to be noted in bacterial colonies under these conditions have already been discussed. Molds should always be examined in this way. Frequently the arrangement of spores on conidiophores, and the branching of the latter, may be better determined by this method than by the preparation of mounts. This is particularly true in those forms in which the conidia are easily detached from their conidiophores. The branching and arrangements of the mycelium may also be observed. Higher-power objectives may be used in this direct examination if a drop of alcohol is placed on the spot to be examined and a cover glass carefully lowered into place. Alcohol removes the air and prevents formation of bubbles, and it is not as likely to cause spores to fall from their stalks as is water. In many cases, better views of mold-spore arrangement may be secured in this way than by any other method. Germination of mold spores may be studied readily by sprinkling the spores to be examined on the surface of agar or gelatin in a petri dish. The germinated spores may be located after the lapse of a suitable period and examined under the higher powers after covering with a glass slip.

Temporary Mounts. Microorganisms are frequently examined by placing a small quantity of the culture in water, physiological salt solution, or broth on a slide, and adding a cover glass. If a mold is to examined, it is often advisable to mount in alcohol first to remove air bubbles. A satisfactory method in the routine study of molds is to place a drop of alcohol on a microscopic slide and tease out the mold hyphae added to it with two needles. A drop of dilute aqueous solution of eosin is added, the mixture allowed to stand for a few seconds, and a cover glass is dropped on. The excess stain is removed by drawing it from under the cover glass by means of a bit of filter paper, water being added from a pipette on the opposite side.

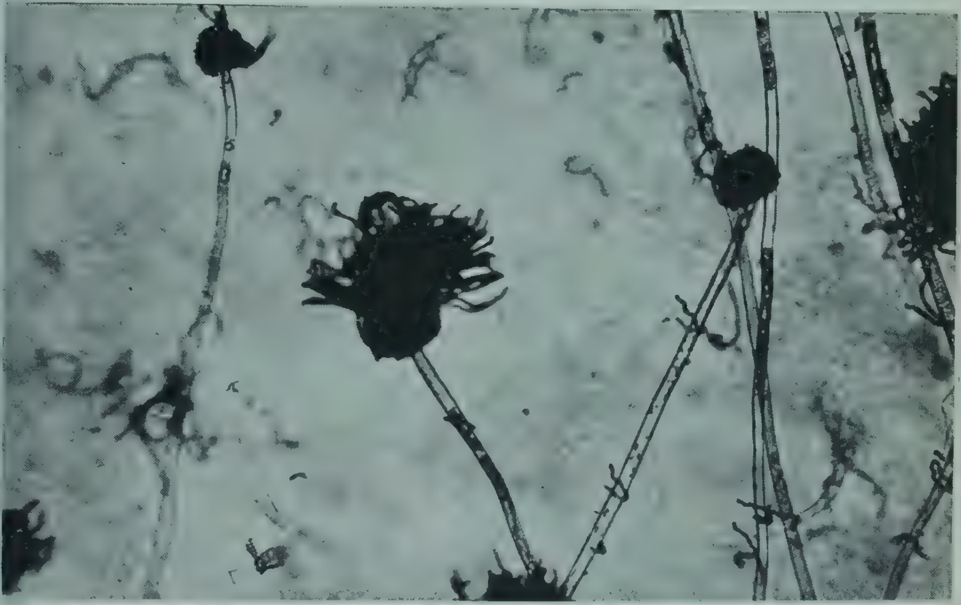

Fig. 15–3. Photomicrograph of *Aspergillus* treated with alcohol and mounted in alcohol. Note that the conidia remain united in chains and many of the chains remain attached to the head. If mounted in water, the conidial chains would break apart.

Many microorganisms are relatively transparent and owe their visibility under the microscope to differences in refractivity between their cell protoplasm and water. With certain of the smaller bacteria in particular, these differences are so slight that considerable care must be used in adjusting the light by means of the iris diaphragms of the substage and the Abbé condenser.

Temporary mounts of this character may be protected from too rapid drying by ringing the cover glass with vaseline.

Hanging Drops. For the determination of motility in bacteria and for the continuous observance of the growth of an organism, the hanging drop may be used. Motility may also be studied in a mount such as was described under the preceding heading. A hanging drop is prepared by placing a drop of the material to be examined upon the center of a cover glass. This is inverted and lowered over the cavity of a hollow ground slide and ringed with vaseline. It is usually best to focus first upon the margin of the drop, as this is more readily visible.

PREPARATION OF STAINED MOUNTS

Reasons for Staining. As has been noted above, it is usually difficult to see unstained bacteria. The primary object of staining is therefore to render organisms more plainly visible. It is of importance also in some cases in revealing structures such as granules within the cell or peculiar construction of the cell walls. Staining also assists in the recognition of capsules and flagella when such are present.

Stains Used. Most of the stains used for bacteriological work are aniline dyes, so-called because of their derivation from aniline ($C_6H_5NH_2$). It is customary to divide these into basic and acid stains. An acid stain is one in which the staining property is in the cation. For example, the acid red dye eosin is sodium eosinate and the ability to stain red is in the anion. Methylene blue is usually methylene blue chloride, and the staining property is resident in the cation. Bacteria are usually stained by the basic stains; certain of the acid stains are useful in studying the molds. Aniline dyes are of practically every known color. Those most commonly used are crystal violet, methylene blue, basic fuchsin, Bismarck brown, safranin, and eosin. A large number of others are used for specific purposes and will be noted later.

Mordants. A mordant is any material which will *fix* a stain, that is, will cause an organism to stain more deeply or retain the stain more firmly than if it were not used. Phenol or aniline when added to certain staining solutions render them more intensive in their action. Mixtures of potassium iodide and iodine, of tannic acid and iron sulfate, and other solutions are used as mordants for particular purposes.

Action of Stains. Different cells and the different parts of cells show wide variations in the stains which they will take up. Quite elaborate theories have been developed to explain the affinities between dyes and the substances stained. For example, some dyes are very soluble in fats and oils; fat globules in a cell may be deeply stained with one of these without the remainder of the cell taking up appreciable amounts of the stain. When plant and animal tissues are stained with certain dyes in sequence and excess dye is removed by washing, certain cells or parts of cells will be found to have retained one dye, another will retain some other color. A theory of staining that will satisfactorily account for all these facts has not been developed. The one which seems to explain a large proportion of the cases emphasizes that, in general, the stainable materials are amphoteric in reaction. Cell proteins and nucleoproteins are able to dissociate both hydrogen and hydroxyl ions, and the relative dissociation is determined in part by the hydrogen ion concentration of the medium. The isoelectric point is that pH of the medium at which the dissociation of hydrogen ions equals the dissociation of hydroxyl ions.

In pH ranges higher than the isoelectric point (on the alkaline side) of the proteins or other amphoteric substances present in the cell, the basic dyes will be taken up. On the acid side of the isoelectric point, the acid dyes are effective.

In general, bacteria and yeasts stain well with basic dyes, and these are most commonly used. In the bacteriological laboratory, one is concerned primarily with shape, grouping and size of cells, more rarely with details of structure.

The composition of the solutions used for staining and directions for their preparation may be found in Standard Methods or in suitable laboratory manuals.[1]

Preparation of Stained Mounts. Stained mounts of bacteria and yeasts are usually prepared by the following method. A small drop of

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[1] The following stains are in common use in the laboratory. For special purposes a great number of others have been described.

Loeffler's Methylene Blue	
Saturated alcoholic solution of methylene blue	15 ml.
Solution of potassium hydroxide 1/1000	45 ml.
Aqueous Solution of Gentian Violet	
Saturated alcoholic solution of gentian violet	2.5 ml.
Distilled water	47.5 ml.
Aniline Gentian Violet (Ehrlich's)	
Saturated alcoholic solution of gentian violet	6 ml.
Absolute alcohol	5 ml.
Aniline water	50 ml.
Aniline water may be prepared by shaking 2 cc. of aniline with 98 cc. of water for several minutes. It should then be filtered through paper until clear. This is practically a saturated solution in water.	
Carbol Fuchsin	
Saturated alcoholic solution of basic fuchsin	5 ml.
Solution of phenol 3–5 per cent	45 ml.
Eosin	
Water-soluble eosin	0.5 g.
Distilled water	100 ml.
Safranin	
Safranin 0 (2.5% solution in 95% alcohol)	10 ml.
Water	100 ml.

water is placed upon a thoroughly clean microscopic slide. By means of a sterile platinum wire or loop, a small portion of the culture to be examined is mixed in the drop of water and spread over the surface of the glass in a uniform layer. If the glass is perfectly clean, there will be no tendency for the water to round up in a drop, but it will adhere to the glass and spread evenly. When the organism is growing in a liquid medium, the water may ordinarily be dispensed with and the preparation made directly with a drop of the nutrient solution. This material is then allowed to dry in the air, or it may be held at a distance above the bunsen burner in such a way that it will not be overheated. As soon as perfectly dry, it is *fixed* by passing film side up two or three times through the flame of the bunsen. This causes the organisms to adhere firmly to the glass, so that they are not easily displaced by later treatment with stains and other reagents. After fixing, the preparation is ready for staining. A drop of the stain to be used is placed upon the surface and allowed to act for a period from a few seconds to as many minutes or even longer, depending upon the organism to be examined and the stain used. In a few cases, it is necessary to keep the stain warm by holding above the bunsen flame until it steams and by replacing the stain as rapidly as it evaporates. The slide is then washed in running water until no more stain comes off. It may then be dried by blotting between filter paper. When completely dry the immersion oil is placed directly upon the film and examined under the microscope.

It is sometimes necessary to use other methods of fixing than heat. For some purposes it is customary to immerse the film in absolute alcohol or expose it to the vapor of osmic acid.

Spore Stain. The spores of bacteria do not ordinarily stain as readily as do the vegetative rods, but when once stained, are not as readily decolorized. Hansen's method is one of the simplest proposed, and usually will yield satisfactory results.

1. Prepare a film of the spore-producing organism. Dry, fix with heat, and stain with steaming hot carbol fuchsin for five minutes. The stain is heated directly upon the cover glass or slide by holding it above the bunsen flame until vapor is seen to rise. It is then moved to one side, and the process is repeated as soon as vapor ceases to be given off.

2. Decolorize with 5 per cent acetic acid until the film is light pink. The time necessary for this will depend upon the preceding

treatment and upon the density of the film; a few seconds is usually sufficient. Then wash in water.

3. Stain for three minutes with Loeffler's methylene blue.
4. Wash in water, dry, and examine.

The spores should be stained red and the bodies of the vegetative rods should be blue.

Flagella Stain. The flagella of bacteria are not visible in stained mounts as usually prepared. Special methods are necessary for their demonstration. These involve the use of mordants which so alter the flagella that when suitable stains are applied they cause not only staining of the flagella but also apparent precipitation of the dye about the filament, increasing thereby its apparent diameter and visibility. Young, actively motile cultures, preferably those not more than twelve to eighteen hours old, should be used. Agar slants are perhaps most satisfactory. A tube containing 5 cc. of sterile water or physiological salt solution is inoculated with a sufficient quantity of growth from the agar slant to produce slight turbidity. This is then kept for an hour in the thermostat at 37.5° C. Two or three drops are placed on a clean cover glass and allowed to dry without mixing or spreading. This is fixed in the flame. Loeffler's method of staining will give very satisfactory results when carefully carried out. Many other methods have been devised.

1. A water bath containing boiling water should be prepared.
2. Prepare the film and fix as already indicated.
3. Apply the following mordant; heat for five minutes over the steaming water bath:

Tannic acid (25 per cent aqueous solution)	10 parts
Saturated solution of ferrous sulfate	5 parts
Fuchsin, saturated alcoholic solution	1 part

4. Wash in water and blot with filter paper.
5. Stain with aniline gentian violet or carbol fuchsin over the hot water bath for five minutes.
6. Wash and examine.

Much care must be used in all details of the process, and repeated trials are often necessary before a satisfactory mount is obtained. With larger bacteria the flagella and their motion may be observed by use of dark-field illumination. The electron microscope gives the best pictures of bacterial flagellation.

Gram's Staining Method. Gram's staining method was first used in the demonstration of bacteria in diseased tissues. By this method certain bacteria were found to retain the stain, whereas the tissues in which they were embedded lost the stain. It was afterwards discovered that bacteria could be divided into two groups, those which would retain the stain when treated by this method, the so-called gram-positive forms, and those which would lose the stain, the gram-negative forms. A statement as to the gram-staining characters of an organism is practically always included as a part of its description. Apparently the gram-negative and gram-positive characteristics of bacteria are closely linked with other characters. They tend to differ in their ability to withstand antibiotics and the antiseptic action of dyes. The gram-negative forms tend to undergo self-digestion (autolysis) more readily. Many theories have been advanced as to the essential differences between gram-positive and gram-negative forms. Recently Batholomew and Umbreit (1944) concluded that in gram-positive bacteria the outer portion of the protoplasm contains ribonucleic acid, and that the presence of this compound is responsible for this staining reaction. When this is removed by digestion with the enzyme ribonuclease, the organisms become gram-negative.

Hucker's modification of the gram-staining technique gives good results.

1. Prepare a film; dry and fix.

2. Stain for one minute with crystal violet-ammonium oxalate solution.[2]

3. Treat with iodine solution one and one-half minutes. This has the following composition:

Iodine	1 g.
Potassium iodide	2 g.
Distilled water	300 ml.

4. Decolorize with 95 per cent alcohol until most of the stain seems to be washed out, or immerse in the alcohol for five minutes.

5. Counter stain in eosin or saffranin for thirty seconds. Wash, dry, and examine.

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[2] *Solution A*

Crystal violet	2 g.
Ethanol (95%).	20 m
Solution B	
Ammonium oxalate	0.8 g.
Water	80.0 m

Mix solutions A and B.

Gram-positive organisms will retain the color of the gentian violet; the gram-negative cells will be stained pink.

Permanent Mounts of Molds. The simple methods that have been described for preparation of permanent mounts of bacteria and yeasts will not suffice for the molds in most instances. Often they show up well without staining. This is particularly true of those forms that have brown or smoky cell walls. These are usually mounted by placing them in a small drop of 50 per cent glycerol on a glass slide, adding a cover glass, removing carefully any excess glycerol, and ringing the cover with asphaltum or microscopic cement both to bind the cover filmly in place and to prevent loss of glycerol. When this treatment causes a collapse of the cell, a 0.5 per cent solution of formalin in water may be substituted. In this case it is absolutely necessary to seal the mount completely or the water will quickly evaporate. Forms that are transparent require staining to bring out details of structure.

CHAPTER 16

Rates of Growth and Death of Microorganisms

EFFECTS OF ENVIRONMENT

The *physiology* of an organism may be defined as including all the interrelationships between the organism and its environment: all the effects of environment upon the organism, and all the effects of the organism upon its environment.

The effects which may be produced upon microorganisms by their physical and chemical environments are of many types. Physical or chemical influences may *accelerate the rates of growth* or increase, they may *accelerate the rates of death* or decrease; they may bring about *changes in the morphology* of the organisms, they may *modify the chemical or physical changes which the organism may produce*, they may *modify the rate at which an organism moves*. These are a few only of the effects which may be manifested.

From a practical point of view we need to know the effects of environment upon rates of growth and death of microorganisms. If under one set of conditions an organism grows slowly and under another set it grows rapidly, we may determine the effect of environment by comparing the rates of growth or the rates of increase. Correspondingly, we may compare the effects of unfavorable conditions by noting the comparative rates of death which they induce. It is apparent, therefore, that we should have methods of measuring or determining the *numbers* of organism or the *amount* of growth. We must also understand what is involved in the term *rate* as applied to increase or to death, and how these rates may be determined.

MEASUREMENT OF NUMBERS OF ORGANISMS OR OF AMOUNTS OF GROWTH

There have been many methods suggested and used for determining at intervals the number of living cells of microorganisms present in a culture medium and for determining the total amount of growth which has developed. Several of the methods more commonly used will be noted.

Estimation of Numbers by Direct Microscopic Count. Three principal methods of determining numbers of microorganisms by direct microscopic observation have been employed.

With the larger microorganisms (particularly with yeasts), it has been found practicable to use a modification of the counting chamber or hemocytometer employed by physicians in estimating the number of cells present in a given volume of the liquid. In this method a known volume of the liquid containing the cells is examined directly under the microscope and the number of cells counted.

Another method is to spread a *definitely measured* small volume of the liquid containing the bacteria over a definite area of a glass microscope slide, followed by drying, fixing, and staining. It is necessary to know the area of the field of the microscopic combination of lenses used for counting. One may then count the number of bacteria or other cells present in a series of microscopic fields. From these numbers one may calculate the number of cells present in the volume of liquid originally spread upon the slide. It is also possible to determine numbers by the method of *proportional counts*. A definite volume of the liquid containing the organisms to be counted is mixed thoroughly with an equal volume of a liquid containing a known number of microscopically observable particles. Frequently blood is used. The mixture is spread on a slide, fixed, and stained. The ratio of the average number of blood cells observed in several microscopic fields to the average number of organisms per field is equal to the ratio of the number of red cells in a cubic millimeter of blood (5,000,000) to the number of organisms per cubic millimeter. These methods of counting microorganisms by direct observation do not usually enable one to differentiate between living and dead cells. Other methods may be required if one is to determine the total number of *living* cells which are present.

Cultural Methods for Counting Living Cells. The most common method of determining the total number of bacterial or yeast cells which may be present is by mixing a definite amount, or dilution, of the liquid containing the organisms with melted nutrient agar or gelatin, then pouring into a sterile petri dish. When cooled, the medium will solidify. This procedure is frequently termed *plating out*. The individual cells are thus fixed in place and under favorable environmental conditions will grow into colonies which may be counted; knowing the dilutions employed in preparation of the plate, one may estimate the number of living cells in a given volume of the material, on the assumption (not always completely justified) that each colony has originated from a single cell. It is important that suitable dilution be employed. For example, if one milliliter of the material is placed in the first plate, one milliliter of a 1–10 dilution in the second, one milliliter of a 1–100 dilution in the third, and so on, and the plates are incubated. The colonies on plates with the larger inocula may be so crowded together that they are difficult to count, indeed the growth or development of some may be altogether inhibited. For estimation of the number of organisms present, choose those plates on which the colonies are neither crowded nor few in number. An estimation of the number of organisms initially present in the material "plated out" is secured by multiplying the number of colonies in a plate by the reciprocal of the dilution used in making the plate.

Methods of estimating numbers by determination of the highest dilution of the liquid which may be inoculated into a suitable culture medium and produce growth have sometimes been employed. For example, one may set up a series of test tubes containing a suitable liquid medium. In the first tube place one milliliter of the material under examination, in the next, one milliliter of dilution 1–10, in the next, one milliliter of dilution 1–100, and so on through the series. The tubes are incubated and observed for growth. If the dilutions were high enough, some tubes at the higher dilutions should remain without growth. Suppose that all the tubes having dilutions of 1–10 to 1–10,000 show growth, those with dilutions of 1–100,000 and above show no growth. The best estimate on the basis of the data would be that the initial material probably contained at least 10,000 bacteria per milliliter and not as many as 100,000 per milliliter. The reliability of the enumeration may be increased by inoculating more tubes of each dilution and recording all those of each dilution which showed growth. By use of data thus secured one may determine the most probable number of organisms present by consultation of tables which have been prepared for this purpose.

Measurement of Increase by Other Methods. It is sometimes practicable to determine the total amount of increase or the amount of growth by measurement of the total volume of organisms as shown by centrifuging the liquid in a suitably calibrated tube, by weighing, by measuring the size of colonies, by determination of the turbidity which is produced when the organisms are grown in a liquid medium, or by measuring the amount of some product of growth. These methods are somewhat less frequently employed than those mentioned above. Details may be found in standard laboratory manuals.

RATES

Rate is defined as the amount of change which takes place in a given length of time. The rate of increase in a bacterial culture, for example, for a given period of time is the increase in the number of bacteria during that interval. A bacterial death rate correspondingly would be the number of bacteria which die in a given length of time. It is quite evident that when bacteria are multiplying, the number of new cells produced in a given length of time will depend upon the number which are present at the beginning of that time. It is frequently advisable therefore to employ the designation *rate of increase per cell* or *rate of death per cell*. This latter rate is often called the *velocity coefficient* of the rate of increase or of decrease. Comparisons of effects of environment are frequently made by noting the velocity coefficients of the rates of change in numbers under the respective conditions.

Rates of Increase. It has already been noted that bacteria commonly multiply by fission, that is, each mother cell divides into two daughter cells. The length of time which elapses between consecutive cell divisions, that is, the length of time that is required for a single cell to grow to its full size and divide to form two individuals, may be termed the *generation time*. It is apparent that the shorter the generation time the more rapidly are the bacteria multiplying. We can therefore judge the effect of various physical or chemical influences upon the growth of an organism by noting any induced changes in the length of the generation time. It is usually not convenient actually to watch the microorganisms under the microscope and to determine by means of a stopwatch the length of time required for a cell to divide in order to get at the length of the generation time. Methods of counting, such as those enumerated above, have been devised so that we may know the number of living bacteria present in a milliliter of liquid at the beginning of any definite period of time and the number at the end of that period. From these data it is possible to calculate the length of the generation time. It must be emphasized that this must be done on the assumption that the conditions do not change materially during the period of observation; in other words, the cells should continue to multiply regularly throughout the period.

Let n be the number of cell divisions, that is, the number of generations which are produced during a given time t. For each initial organism, at the end of one generation period there will be two organisms, and at the end of the second generation period four organisms, and at the end of the third period eight organisms. It is apparent, therefore, that the number of organisms originating from a single cell will be at the end of the first period 2^1, at the end of the second period 2^2, at the end of the third period 2^3, and at the end of the nth period 2^n. If we represent by B the initial number of organisms, then the number at the end of the nth generation period will be $B2^n$.

If b represents the number of bacteria at end of time t it is evident that

$$b = B2^n \tag{1}$$

The number of generations (n) that will develop in time t must equal the ratio between the time (t) and the generation time (g), or

$$n = \frac{t}{g}$$

and

$$b = B2^{\frac{t}{g}} \tag{2}$$

If one plots the numbers of bacteria against time in accordance with equation (2), what type of a curve is secured? Figure 16–1A is the graph of such a curve in which the initial number of bacteria (at $t = 0$) is taken as 2 and the generation time as 15 minutes. It is apparent that the curve rises more and more rapidly; the more bacteria are present the more rapid the increase.

Additional information may be secured by solving equation (2) for $\log b$.

$$\log b = \log B + \frac{\log 2}{g} t \tag{3}$$

Equation (3) is in the form of an equation of a straight line. When $\log b$ is plotted against time, a straight line is secured in which the intercept with the y axis is $\log B$, and the slope k of the line is

$$k = \frac{\log 2}{g}$$

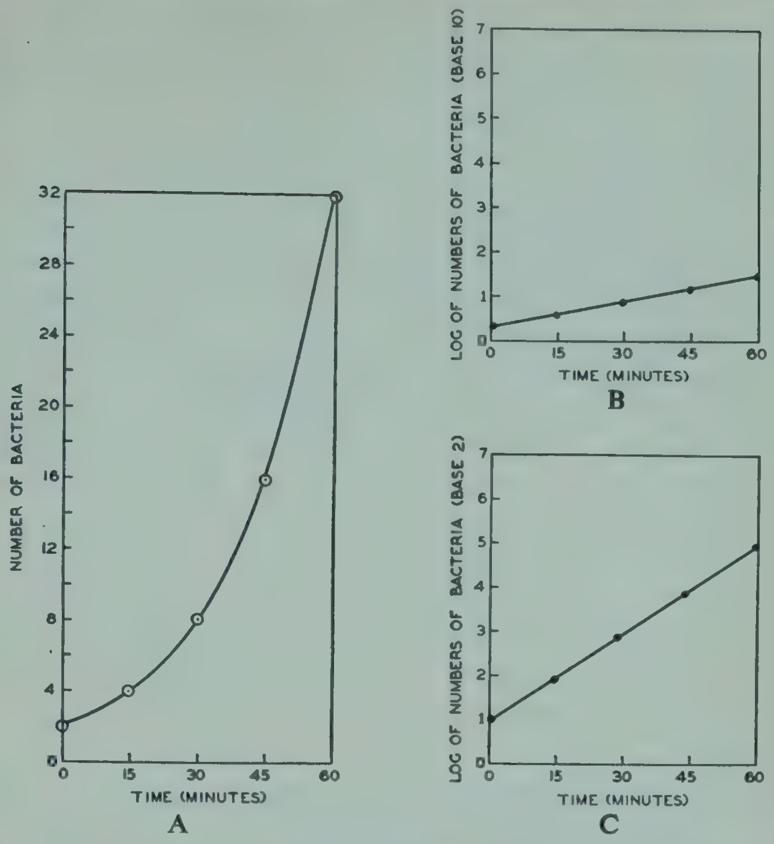

Fig. 16–1. Graphs illustrating the increase in the number of bacteria during the period of logarithmic growth. A. Graph of numbers of bacteria against time, beginning with 2 bacteria, with a generation time of fifteen minutes. At the end of an hour the number of bacteria has increased to 32. B. Graph of the logarithms (base 10) of the numbers of bacteria against time, yielding a straight line. The slope of this line is positive, it is the tangent of the angle which the line (extended) makes with the *X* axis. This slope, that is the steepness of the line, is the best measure of the rate of increase in numbers in the culture of bacteria. C. Graph of the logarithms (base 2) of the numbers of bacteria against time. Note that the slope of this line is greater than that secured when base 10 logarithms are used.

Obviously the numerical value of k will differ with the choice of the base of logarithms, as, for example:

With logarithms to the base 2,

$$\log_2 2 = 1 \quad \text{and} \quad k_2 = \frac{1}{g}$$

With logarithms to the base 10,

$$\log_{10} 2 = 0.301030 \quad \text{and} \quad k_{10} = \frac{1}{3.3g}$$

Figure 16–1C is a graph of $\log_2 b$ against time, and Fig. 16–1B a similar graph of $\log_{10} b$, with the values for B and g those used for Fig. 16–1A. In each case a straight line is produced, but with marked difference in the slope.[1]

It is evident that the steeper the slope of the line, the greater the value of k, and the more favorable the conditions for growth. If two or more cultures of the same organism are exposed to environments differing in only one factor, such as temperature, plots of the numbers of bacteria against time would yield a series of straight lines with different slopes. The ratio between the slopes of any two lines is an index to the effects of the two environments on the rates of growth.

Whenever a plot of the logs of the numbers of bacteria against time yields a straight line, i.e., when the values of k and g are constant, the the culture is said to be in the *logarithmic period* of growth.[2]

Growth Phases. When viable bacterial cells are introduced into a favorable environment (medium), they begin to grow and multiply. After a time the culture medium becomes less favorable for growth, and the cells divide less and less rapidly; they begin to die. It is possible, therefore, to recognize several distinct growth phases in a bacterial culture.

One may follow through the consecutive growth phases by inoculating a tube of a suitable culture medium with some resting bacteria (or with bacterial spores) and counting from time to time by any suitable method the number of living bacteria present. For some time the

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[1] Inspection shows that the value of k (the velocity coefficient of the rate of growth, or the slope) when logarithms to base 2 are used is the reciprocal of the length of time required to double the number of organisms, and its value when $\log_{10}$ is used is the reciprocal of the length of time required for the organisms to increase to a population ten times the initial.

[2] Another somewhat more generalized statement relative to rates of increase may be developed as follows. The rate of increase $\frac{db}{dt}$ is proportional at all times to the number of organisms present;

$$\frac{db}{dt} \propto b \quad \text{or} \quad \frac{db}{dt} = kb \tag{5}$$

in which k is a proportionality constant, or the velocity coefficient of the rate of increase. Integration of equation (5) yields

$$\ln b = \ln B + kt; \quad k = \frac{\ln b - \ln B}{t}$$

and

$$b = Be^{kt}$$

in which e is the base of natural logarithms.

The velocity coefficient of the rate of increase in this case is the reciprocal of the length of time required to multiply the number of organisms by $e = 2.7182$

number of cells will remain constant, for cells or spores in a resting stage require time for the cells to germinate and begin to multiply. The time required for bacterial cultures to develop to the point where all cells are growing and multiplying at the maximum rate is termed the *lag* period. After the close of the lag period, the cells will be multiplying at their maximum rate for the particular environment. During this period the relations discussed in the preceding section under "Rates of Increase" will hold. This period is usually called the *logarithmic growth phase,* or the phase in which the generation time is at a minimum (and the velocity constant is at a maximum). After a time the rate of growth slows down as food becomes less abundant, and the injurious growth products of cell growth accumulate. This period of slower growth may be termed the phase of *negative growth acceleration.* Later the bacteria will cease dividing, and a *stationary phase* ensues. Eventually the bacteria will begin to die, and the *death phase* is initiated.

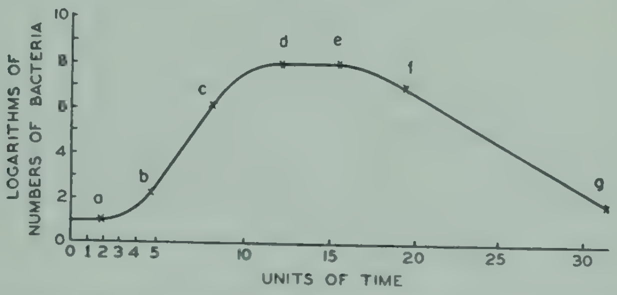

Fig. 16–2. Graph of the growth stages or phases in a culture of bacteria. To *a*. Phase of no growth (or no increase in numbers). *a-b*. Phase of positive growth acceleration; the slope of the plotted line increases with time. *b-c*. Phase of logarithmic growth, the slope of the line is constant and positive. *c-d*. Phase of negative growth acceleration. The average generation time is increasing. *d-e*. Maximum stationary phase, no increase in numbers. *e-f*. Phase of accelerated death. *f-g*. Phase of logarithmic death, a straight line with a negative slope.

Cells of microorganisms may show considerable variation in resistance to unfavorable conditions depending upon the growth phase in which they are studied.

Rates of Death. When organisms are placed under sufficiently unfavorable conditions, they cease to multiply and begin to die. In many of the cases which have been carefully studied, the bacteria die in accordance with a definite law, which may be stated as follows. With a given kind of organism under uniform conditions, the number of bacteria present in a culture will always be reduced by one-half in equal periods of time; that is, no matter how many bacteria there are at the beginning of a definite period of time, one-half that number will be alive at the end of the period. For example, two cultures, one containing a million bacteria and the other a thousand bacteria, may be

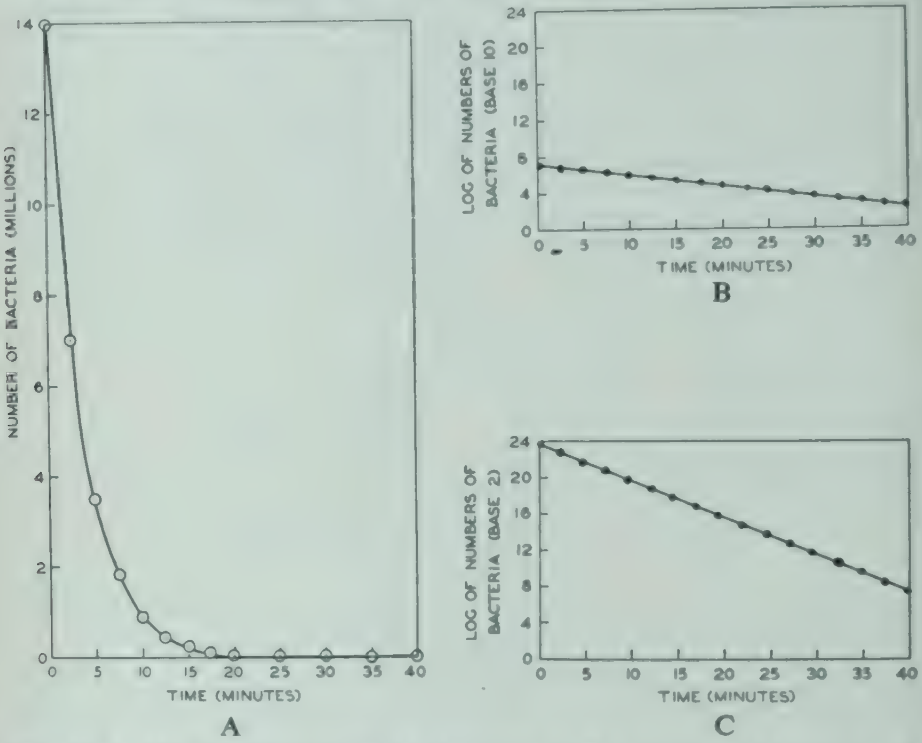

Fig. 16–3. Graphs illustrating the decrease in the number of bacteria when placed under unfavorable conditions on the assumption that the rate of death is constantly proportional to the number of living organisms, that is, that the death curve is logarithmic. A. Graph of the numbers of living bacteria against time, beginning with 14 million living organisms and on the assumption that 50 per cent of the living bacteria die off in each succeeding two and one-half minute interval of time. B. Graph of the logarithms (base 10) of the number of survivors against time. The slope of the straight line is negative, being the tangent of the angle that the line makes with the *X* axis. The slope of this line, the velocity coefficient of the rate of death is a measure of the rate of death. C. Graph of the logarithms (base 2) of the number of survivors against time. Note that the slope of this line is greater than that of B.

subjected to the same unfavorable conditions. It is found that at the end of a definite period of time, say ten minutes, the bacteria in the less concentrated suspension have been reduced to 500. It will be found that in the same period of time, the number of bacteria in the other culture has also been halved, that is, there are 500,000 bacteria left. Another way of stating this is that during each equal interval of time a definite percentage of those bacteria living at the beginning of the period will be killed. If we wish to compare unfavorable conditions as to their effect upon the death of microorganisms, we may compare the lengths of time required to reduce the numbers of bacteria by a definite percentage, say one-half, or in some cases 99 per cent. If at one temperature, for example, half of the bacteria are killed in ten minutes and at another temperature one-half the bacteria are killed in five minutes, it is evident that the second temperature is far more destructive than the first. It will be noted that the time required to kill half the bacteria is mathematically the converse of the generation time. When organisms die in this fashion, the designation logarithmic death phase is used.[3] When the logarithms of the numbers of survivors are plotted against time, a straight line is produced with a negative slope.

The statement above outlined does not hold good for all bacteria under all conditions. For example, a plot of the log of surviving spores against time frequently does not yield a straight line. When it does, however, it is possible to compare environmental effects on death rates by direct comparison of the several velocity coefficients of the rate of death.

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[3] When bacteria die as noted above, it may be shown that the rate of decrease in numbers is constantly proportional to the number of living bacteria present and that there is a constant rate of death per cell, or velocity constant, which may be determined at the beginning and end of a definite period of time from the following relationship:

$$K = \frac{2.3}{t} \log \frac{B}{b}$$

CHAPTER 17

Effects of Physical Agencies on Microorganisms

The principal physical factors influencing growth, development, and inhibition and death of microorganisms are *moisture* and *desiccation, osmotic pressure, surface tension, radiation, temperature, mechanical pressure, electricity*, and *vibration*.

Moisture. The optimum moisture condition for most yeasts and bacteria is saturation of the environment. Molds frequently develop best with less moisture. Complete *desiccation* (drying) at temperatures above freezing may kill many organisms, particularly certain of the disease-producing forms such as the typhoid bacillus. On the other hand, the spores of bacteria, yeasts, and molds may withstand drying and remain viable for years. Some bacteria and yeasts that produce no spores are also very resistant to desiccation.

Drying stops all growth and activity of the microorganisms that may be present in any food or other readily decomposable material. It is therefore of importance as a means for preservation of foods and in the prevention of decay in general.

Why should drying kill organisms? Water is essential to the metabolism of the cell. A dry cell cannot grow. Some cells when dried apparently remain dormant and resume growth when moisture is again supplied. Other kinds of cells are killed rather promptly. Apparently resistance to desiccation is in part an inherent character of the organism. Further, the degree of resistance is determined in part by the growth phase. Bacteria which are in the period of most rapid multiplication (the logarithmic growth stage) are most susceptible, resting cells and spores are more resistant.

The conditions under which the drying takes place are important in determining death rates. For example, certain bacteria if dried from a suspension in water will be quickly killed, but if surrounded by gelatin, mucus, or certain gums when drying, they retain their viability for a long period of time.

Dried organisms usually remain viable much longer if they are kept from contact with oxygen, as by being sealed in a vacuum or in an inert gas such as nitrogen.

Advantage is taken of these facts in the long-term preservation of pure cultures of bacteria and other microorganisms for later use. The maintenance of a stock of pure cultures needed from time to time is very time-consuming, if the cultures must be regularly transferred to new tubes of nutrient media. In addition to the great amount of labor involved there is always the possibility of contamination with other organisms, also the likelihood with some species that a mutant arising by variation in the original may be picked up, yielding a culture differing in some characters from the parent form. It is now customary in many laboratories, particularly those maintaining large collections such as that of the American Type Culture Collection, to use the process of *lyophilization,*[1] which involves drying frozen organisms on a suitable substrate, and in a partial vacuum. Small quantities of the organisms or materials to be lyophilized are distributed in ampuls or other suitable glass containers, immersed in a mixture of dry ice (solid carbon dioxide) and ethanol (temperature about —78° C.), quickly frozen, and attached to a vacuum pump which promptly dries the tube contents. Tubes are sealed and kept refrigerated. Under these conditions many, indeed most, species of organisms remain viable for long periods of time. When transferred to a suitable medium, they grow normally and show the characters of the original culture. This process is used extensively not only for preservation of bacteria, but also for viruses, enzymes, vaccines, and the many components of blood serum.

Osmotic Pressure. In the discussion of morphology of microorganisms in Chapter 4, the outer differentiated layer of protoplasm lying just within the cell wall was described as a semi-permeable membrane. The normal cell generally has within this membrane a higher content of solutes than is to be found in the solution in which the cell is growing. This leads to an excess osmotic pressure on the interior of the cell. The protoplasm is firmly appressed to the cell wall by this internal pressure and the cell is said to be *turgid*. When a cell is placed in a solution having a higher content of solutes than has the protoplasm and the solutes do not readily pass into the cell, conditions are reversed; water leaves the cell and it shrinks in size. If the difference is great enough, this shrinking continues until the plasma membrane separates from the cell wall due to the contraction of the protoplasm. This process is called *plasmolysis*.[2] If the difference is not too great, the cell gradually adjusts itself to the new conditions, regains its turgor, and continues growth. If the difference is greater, the cell remains permanently plasmolyzed and is killed. Transfer of a cell grown in a solution containing a high concentration of solutes to distilled water may bring about the reverse of plasmolysis, a swelling of the cell, sometimes followed by a bursting of the cell wall and escape of the contents. This is called *plasmoptysis*.[3] If a solution surrounding a cell has such concentrations of solutes that it causes the cell neither to swell nor to shrink, if, in other words, the solution and the protoplasm are in osmotic equilibrium, the solution is said to be *isotonic*. If it causes swelling it is *hypotonic*, if it causes shrinking it is *hypertonic*.

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[1] *Lyophilic* is a designation applied to colloidal substances such as proteins which have affinity for water or other liquids in which they tend to disperse or dissolve. The adjective comes from two Greek words, *lyo,* to dissolve, and *philus,* loving, and has the meaning of dissolving readily. The drying of certain colloids brings about an irreversible change; some originally dispersible in water no longer dissolve. To *lyophilize* means to treat the colloid in such fashion that when dried it will still readily go into solution. It was found that many proteins when frozen and then dried remained lyophilic. The drying of many organisms at higher temperatures apparently so changes the proteins that they no longer take up water normally—they are *denaturated.* The cell cannot revive. Lyophilization dries the organism under such conditions that it will revive when water is added. It seems that freezing prevents any motion of the protein molecules during the drying process; they cannot change; and denaturation is avoided.

Bacterial cells are relatively refractory to plasmolysis; it is most easily demonstrated in members of the genus *Vibrio*. This phenomenon, as well as plasmoptysis, can be readily observed in molds.

With many solutes one may readily secure concentrations too great to permit the growth of organisms. Advantage is taken of this fact in the preservation of foods (as syrups, jellies, and salted foods). Some organisms can adjust themselves to a very high concentration of sugar, as evidenced by the development of molds on jellies and preserves. Some yeasts are known which grow by preference or only in high concentrations of sugar. They are sometimes termed *osmophilous* (or *osmophilic*). As will be discussed later, some organisms are favored by high concentrations of salts in the medium; they are *halophilic*.

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[2] From the Greek *plasma* and *lysis*, meaning literally the loosing or withdrawal of the plasma. In most other words ending in *lysis* the meaning is a derived one, solution, as in *cytolysis*, a dissolving of the cell.

[3] From the Greek *plasma* and *ptysis* (a spitting out, casting out), meaning the extrusion of protoplasm.

Surface and Interfacial Tension. Liquids are always bounded by surfaces which have some of the characteristics of membranes. Liquids tend to maintain a minimum surface area. The surface between a liquid and gas, as that between water and air, behaves something like an elastic (rubber) membrane, differing however in that its ability to stretch is not limited.

A drop of water in air tends to become spherical because the surface film is under tension and the sphere has the least surface for a given volume. It becomes spherical for the same reason that a toy balloon becomes spherical when inflated. This tendency to act like a stretched membrane is evidence of *surface tension*.[4] It is measured usually in dynes per square centimeter.

Many methods of determining surface tension have been devised. One often used is to determine the force required to separate a platinum ring from the surface of the liquid. The apparatus set up for this determination is called a *tensiometer*.

The surface tensions of various liquids show wide differences. At temperatures approximating 18° C., pure water has a surface tension of 73.0 dynes, glycerol 65.2 dynes, ethyl alcohol 21.7 dynes. Increase in temperature decreases the surface tension. For example, the surface tensions of water at 0° C. and 90° C. are 75.6 dynes and 62.9 dynes respectively.

Particularly significant in bacteriology is the change which takes place in surface tension due to the presence of solutes in the liquid. Many substances decrease the surface tension when dissolved in water. For example, at 15° C. pure water has a surface tension of 72.2, with 10 per cent ethanol it is 51.2, and with 50 per cent ethanol 29.1. Any substance which lowers surface tension tends to accumulate in the surface. When a substance in solution is more highly concentrated in the surface film than in the remainder of the liquid, it is said to be *adsorbed,* and the phenomenon of surface accumulation is termed *positive adsorption*.

Surface films form on liquids not only in contact with gases but also in contact with solids and other liquids. The tension of the surface in contact with a gas is *surface tension,* the corresponding tension against a liquid or a solid is termed *interfacial tension*. Surface tensions are much more easily measured than are interfacial tensions. However, it is found that many of the factors influencing surface tension likewise influence interfacial tension. These last are of special interest because of their importance at the surfaces of microorganisms. Substances in solution may tend to collect on the interfacial surfaces, i.e., be adsorbed by the organisms.

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[4] Technically, surface tension may be defined as the force required to overcome the tendency of the liquid to maintain a minimum surface area, or as the amount of work required to produce a new surface of unit area at constant temperature.

Changes in surface tension may affect cultures of microorganisms in several ways. They may influence the type or manner of growth, dispersion, or clumping of the cells of the organism in the medium, the morphology of the cells, the ease with which oxygen may diffuse into the medium, the staining properties, and even the virulence.

In a study of the effect of chemicals which depress surface tension upon the growth of microorganisms, care must be used to differentiate between such effects and the chemical effects upon the organism that may be quite independent of surface tension.

In recent years there has been much interest in a group of compounds which actively depress surface tension, the so-called *wetting agents*. These are compounds which adsorb to interfacial surfaces and greatly increase the ability of water to wet them. Among these are two compounds known under their proprietary names as Tween 80 and Triton A_{20}.[5] Dubos (1949), for example, notes that in a culture medium containing 0.01 to 0.05 per cent Tween 80, the bacillus of tuberculosis will grow with cells well separated, while in usual culture media they tend to grow together in clumps. Other types of wetting agents are effective antiseptics (as zephiram), and still others, as sodium lauryl sulfate, are valuable additions to certain selective media.

Light and Radiation. The light which is useful for vision constitutes only a small fraction of the whole spectrum. Visible light is *radiation* made up of *electromagnetic waves* with wavelengths within certain narrow limits. Not only the wavelengths of visible light, but also radiations of longer and of shorter wavelengths may be of significance in microbiology. The characteristics of any radiation are ordinarily determined by its wavelength, though other related units, wave number and frequency, may be used for special purposes. The wavelengths of radiations may be relatively short and measured in angstroms to those which are relatively very long and measured in meters. In Fig. 17–1 is given a diagram indicating the wavelengths of the spectrum and the names that have been given to important portions, and to the several units of length commonly employed. Note that measurements are given in powers of ten, and the scale is therefore a logarithmic one.

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[5] Tween 80 is one of the polyoxyethylene derivatives of sorbitan esters of long chain fatty acids. Triton A_{20} is an arylalkyl polyether of phenol.

Not all wavelengths of radiant energy affect microorganisms, or indeed, living cells in general. In order to be effective the radiant energy must be absorbed by some part of the protoplasm of the cell. Protoplasm is transparent to most wavelengths, which pass through unabsorbed and have no effect. Infrared light if absorbed, in general, increases the temperature; whether or not it is injurious or proves stimulating depends upon the temperature produced. Radiation in certain other bands of the spectrum may be absorbed by certain cell components and may produce significant chemical changes, even destroying the cell.

Some microorganisms produce colored cell walls which absorb certain lights rays and prevent their penetration to the protoplasm on the inside of the cell. Such protective coloration is

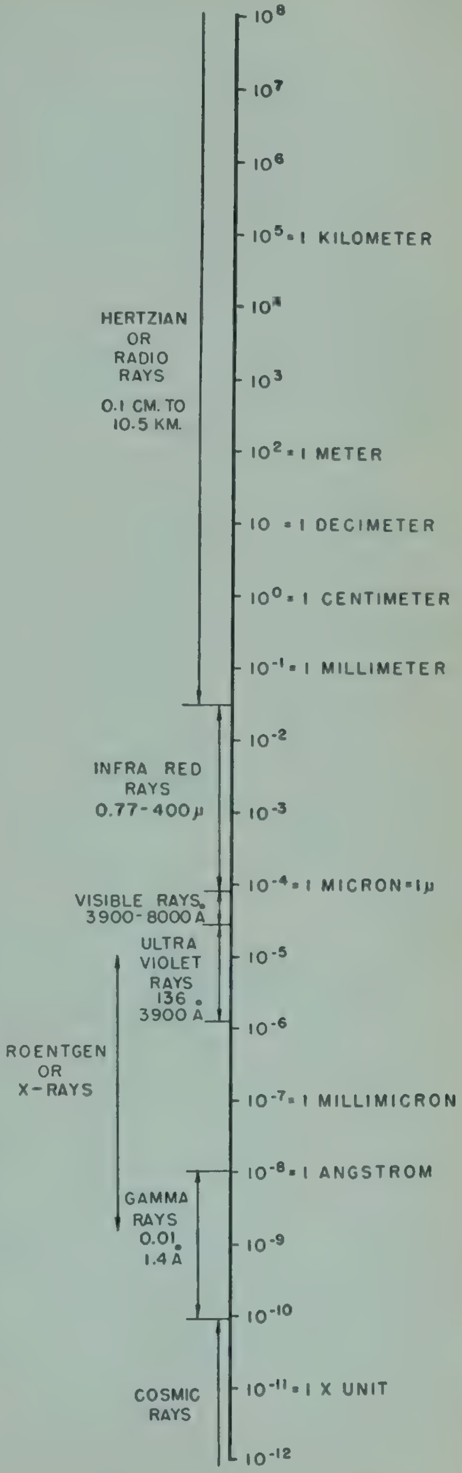

Fig. 17–1. Diagram of the wavelengths of the spectrum from Cosmic to Hertzian. Note that the scale is logarithmic, each unit length on the scale is ten times as large as that next below. Note also the comparatively narrow band of the visible rays.

common in certain groups of molds, particularly the *Dematiaceae*.

A few bacteria produce pigments within the protoplasm which absorb certain light rays and enable the cell to utilize the energy in useful cell processes. All green plants, including the green and purple bacteria, contain the pigment chlorophyll or a closely related compound. These pigments take up the red rays of the visible spectrum, and the energy thus acquired is used in carrying forward the assimilation of carbon dioxide. The manufacture of compounds useful in building up the cell from carbon dioxide is called *photosynthesis*. Chlorophyll is found in only a few bacteria and protozoa, never in the molds and yeasts.

Light may cause certain modifications in growth of microorganisms. Some molds, for example, are stimulated to production of spores by light. This is readily seen in cultures of certain molds growing in a petri dish. If kept in the dark, spores are not formed; if placed in the light they will be produced. If kept for several days where light will strike during the day, the colony may show concentric rings of spores, one ring for each day of illumination.

In a few molds the direction of growth of certain of the hyphae, particularly the fertile or spore-producing hyphae, is apparently determined by the incidence of the light rays. These hyphae grow in the direction of the source of light, just as a green plant in a window tends to grow toward the light. The phenomenon is called *phototropism*.[6]

Many motile organisms that contain a photosynthetic pigment will swim toward the light (*phototaxis*). If growing in a tube of suitable liquid medium with light coming from one side, they will collect on that side of the tube.

Radiation by certain wavelengths may induce hereditary changes or mutations in cells. The x rays and some ultraviolet rays are particularly significant in that they are absorbed by certain protoplasmic elements, particularly the nucleoproteins. Irradiation of microorganisms and viruses with appropriate dosages has been found greatly to increase the rate of mutation and has become an important tool in genetic studies.

Certain rays are also highly destructive to cells. This is particularly true of x rays. In the region of the visible spectrum not all rays are equally injurious to cells. The red and yellow rays are relatively inert.

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[6] From the Greek, *light directed*.

whereas the blue and violet rays may be more destructive. The spectrum extends beyond the violet and indigo into the ultraviolet, rays not recognized by the human eye. These are by far the most destructive. This germicidal action of ultraviolet rays has been utilized practically in certain devices for the purification of water. The Cooper-Hewitt mercury vapor arc lamp has been used for this purpose. This lamp consists of a cylinder of glass or quartz exhausted of air, containing some mercury, and with electric terminals at the ends. The light issues from the mercury vapor arc between these two poles. Glass does not permit the ready passage of ultraviolet ray; hence it is replaced by quartz in lamps designed for sterilizing purposes. The water to be sterilized is allowed to flow past the lamp and is exposed to the rays at short range for a few seconds. The organisms present are destroyed by this exposure.

Temperature. It is a general rule in chemistry that an increase in temperature increases the rate[7] at which a chemical reaction takes place. Very commonly (though by no means universally) the rate doubles or trebles for each ten-degree increase in temperature. Inasmuch as the life processes of a cell are largely chemical or based upon chemical changes, it might be inferred that increases in temperature would similarly increase biological rates of change. In fact, within certain limits fixed for each kind of organism and set of conditions, an increase in temperature does increase the rate of growth. For every cell, however, there is apparently a temperature which yields the most rapid growth; at higher temperatures the rate of growth decreases, and at sufficiently high temperatures ceases altogether. In other words, for every living cell there is an *optimum,* a *maximum,* and a *minimum* growth temperature and a *growth temperature range*. These special temperatures are sometimes termed the *cardinal points*. At temperatures higher than the maximum, microorganisms are destroyed; the higher the temperature above this maximum, the more rapid the destruction.

Optimum Growth Temperature. The optimum temperature for an organism may be defined as that temperature at which it grows most rapidly, that is, that temperature at which the generation time is least. The optimum is not a fixed temperature for a particular species but varies with changes in the environment. It may be modified, for example, by an alteration in the composition of the nutrient medium in which it is grown. Usually, however, the change in optimum temperature with variation of the other environmental conditions is not great enough to interfere with the classification of microorganisms into the three groups noted below.

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[7] That is, the rate per unit weight of compound being transformed.

Thermophilic bacteria are those that develop best at relatively high temperatures, usually above 45° to 55° C. These organisms have been repeatedly isolated from the waters of hot springs, from the interior of decaying heaps of compost or manure, from fermenting ensilage, from soil, and from the intestinal contents of man and animals. Many of them are spore-bearing bacilli. A few of them are of some practical importance as in causing undesirable changes in canned foods when stored at high temperatures.

Psychrophilic or *cryophilic* organisms are those that grow best at relatively low temperatures, usually below 10° C. They are commonest in cold waters such as those of springs, wells, and the depths of lakes or the ocean. Some of these may be of importance in the decay of foods in cold storage.

Mesophilic organisms are those whose optimum temperatures are between the two extremes just noted. They may be divided into two groups, those found in the bodies of man and animals in health or disease and having an optimum temperature of about blood heat (37.5° C.), and those having optima somewhat lower. The decay-producing and putrefactive bacteria frequently have optima between 20° and 35° C. Yeasts, in general, grow best between 20° and 30° C., and molds have similar or even lower optima.

It should be noted that the optimum temperature is not necessarily the temperature at which the organism will bring about a maximum amount of change. Less acid, for example, may be produced at the end of a certain period by an organism kept at its optimum than when maintained at a lower temperature. It will later be noted that the efficiency of antiseptics depends in part upon temperature; a small amount of acid at a high temperature may have a more deterrent effect upon growth than a larger amount at a low temperature.

Minimum Growth Temperature. The minimum growth temperature of an organism is the lowest temperature at which growth will occur. The minimum for any true thermophile is above 40° C. The minimum for some pathogenic bacteria is but two or three degrees below the optimum. In most cases, however, it lies much lower, usually at 8° to 10° C. Some can develop at still lower temperatures. Freezing at once stops multiplication in all cases, probably because ice is dry, and the effect is much as in desiccation. When solutes are present in sufficient quantity aqueous solutions may be lowered to a temperature considerably below 0° C. without freezing. Under these conditions some organisms continue to multiply slowly. It is evident, then, that some foodstuffs cannot be preserved by cold indefinitely at a temperature above the point of freezing of the liquids contained.

Maximum Growth Temperature. The maximum growth temperature is the highest temperature at which growth and multiplication of an organism can take place. In most cases, but by no means in all, the maximum temperature is but a few degrees higher than the optimum. The maximum temperature for some of the thermophilic bacteria is nearly 80° C. This temperature is so high that we must think of their protoplasm as differing from that of other cells, for most native proteins coagulate on exposure to this temperature. The maximum for most pathogenic bacteria lies between 40° and 50° C. Growth at high temperatures sometimes causes a decrease in virulence or disease-producing power. The maximum for many yeasts is between 30° and 40° C. Very few molds develop well at body heat.

The *growth temperature range* of an organism is the number of degrees difference between the minimum and the maximum. This is very small with some bacteria, particularly certain of the pathogenic forms. Some species will not develop unless kept within two or three degrees of body temperature; in most cases, however, the range is much broader.

Freezing does not commonly destroy microorganisms at once. When frozen, they gradually decrease in numbers. Temperatures lower than freezing often do not seem to have any additional effect. Milk containing lactic acid bacteria may be exposed to the temperature of liquid air and, when thawed and kept for a time at room temperature, will sour normally. Sterilization of food products by freezing is therefore not practicable. The gradual decrease in numbers of living organisms in frozen materials is of considerable importance in that it is undoubtedly one of the safeguards in the use of ice. Bacteria, particularly the disease-producing forms, do not persist indefinitely. This decrease is not even more striking with frozen milk products such as ice cream.

Thermal Death Point. A thermal death point is sometimes defined as that temperature which in a given length of time will kill a particular organism. This definition, however, is hardly adequate. We have seen that all the organisms in a culture do not die instantaneously upon being subjected to unfavorable conditions, but that under a definite set of conditions, there will be a definite rate of death. Theoretically it would be better to designate the *rate of death* under certain standard conditions at a definite temperature, rather than to use the term "thermal death point." It has also been suggested that the term "thermal death time" be used to designate the length of time required to kill all the cells at a given temperature. The destruction of bacteria at a given temperature is influenced by several factors.

First, the phrase *rate of death* implies a change of numbers of bacteria with time; therefore, in determining a thermal death point the *time of exposure* must always be known. A lower temperature for a longer time may be as efficient as a higher temperature for a shorter time. This is a matter of importance in the sterilization and pasteurization of foods where it is necessary to kill all bacteria or all cells of certain types, but where heating to too high a temperature will injure the flavor. The time commonly used in *thermal death point* determination is ten minutes, and where time is not specified, this period is usually understood. It is evident from preceding discussions that the time of exposure necessary to sterilize is also dependent upon the *number of bacteria* present in the beginning. It will take a longer time to kill all of a large number of bacteria than all of a smaller number.

Second, the thermal death point (and rate of death) will depend upon the *amount of moisture* present. Moist heat is much more efficient than dry heat. Probably the explanation for this is to be sought in the difference in coagulation (denaturation) temperatures of moist and dry proteins. The albuminous protoplasm of the cell is not readily coagulated when dry. This fact is well illustrated by the following data relative to egg white.

Egg albumin with 50 per cent water coagulates at 56° C., with 25 per cent water at 74°–80° C., with 18 per cent water at 80°–90° C., with 6 per cent water at 145° C. It is evident therefore that in the sterilization of dry objects, as laboratory glassware, it is necessary to use a relatively high temperature as 140° to 150° C. for an hour, while moist heat, as in the autoclave, is even more efficient at 120° C. for ten minutes. Boiling in water for ten minutes will kill everything but the more resistant spores.

Third, the *reaction and composition* of the medium in which the organism is heated have a marked influence on the thermal death time. It is much easier to sterilize an acid fruit, such as the strawberry or tomato, than the more nearly neutral vegetables, such as peas or corn. Acidity in particular renders heat much more effective. It is necessary in comparative work, therefore, to use media having uniform reaction and composition. The term acidity as here used, means, of course, the actual acidity, or hydrogen ion activity. Other substances may also be important. For example, the thermal death rate of bacteria is lower in cream than in whole milk, and lower in the latter than in skim milk.

Fourth, the *presence of spores* causes an organism to have two thermal death points, one for the spores and a lower one for the vegetative cells. Boiling for an hour will not certainly destroy all spores. Use of the autoclave and temperatures of 110° to 120° C. are necessary if they are to be killed in a short period of time. The spores of certain of the thermophilic bacteria are particularly difficult to destroy. They are of considerable importance, as in the commercial canning of corn.

Fifth, the *specific character of the organism* must be taken into consideration. There are evidently intrinsic differences in the protoplasm in different species. Many organisms are destroyed rapidly at temperatures of 55° to 60° C.; others require much higher temperatures. The careful determination of the thermal death time for all pathogenic bacteria is evidently desirable, for upon this determination must rest the efficiency of pasteurization of milk in preventing the spread of disease.

It should again be emphasized that microorganisms held at temperatures above the maximum growth temperature are dying and that the rate of death increases rapidly with the increase in temperature. It has been found with spores that the death rate is increased five to eight times by each increase of ten degrees in temperature; for example, if a temperature of 100° C. for thirty minutes is required to kill a certain number of spores, a temperature of 110° C. for less than six minutes would probably prove equally effective.

It may be noted that sometimes a practical sterilization by heat may be effected without actually destroying all the bacteria present, provided the nature of the medium in which the organisms are present or the conditions under which it is kept are such as to prevent growth.

Mechanical Pressure. Attempts have been made to destroy bacteria by means of mechanical pressure. For example, investigators have tried to kill the bacteria by filling tin cans with food to be preserved, then immersing the sealed cans in water and subjecting them to hydraulic pressure. It was not found practicable to apply pressures sufficiently high to kill all the microorganisms present, particularly the spores of bacteria. It has been found possible by use of pressure of many thousands of pounds to the square inch to kill nonsporulating organisms.

Electricity. Electricity has apparently little direct effect upon bacteria; the passage of an electric current of low voltage through a suspension of bacteria is not directly injurious to the cells. Under certain conditions, however, the electric current may bring about the formation of compounds that act as disinfectants. Advantage is taken of this fact in certain cases in the sterilization of sewage effluents or other solutions containing the chlorides of sodium, calcium, or magnesium. When a current of sufficient tension (at least 2.5 volts) is passed through such a solution, chlorine gas appears at one electrode, alkalies at the other. This process of electrolytic disinfection has proved to be a relatively efficient method of freeing water from microorganisms.

Vibration. Sound waves are transmitted as vibration in matter. They are frequently classified as *sonic*, those of a wavelength which can be heard by the human ears, and *ultrasonic*, those whose wavelengths are so short as to be inaudible The line of demarcation between sonic and ultrasonic sound is not well fixed, frequently 10,000 vibrations per second is taken as the dividing line. Sonic waves of sufficient intensity, 8900 cycles per second, have been found to destroy red blood cells and some bacteria. But effects produced by ultrasonic waves are much more drastic.

High-frequency waves from 100,000 to more than 2,000,000 cycles have been produced by causing quartz crystals to expand and contract in unison with a periodic electrical field. Such vibrations are highly disruptive to cells, breaking them into pieces rapidly, and destroying them. Such effects have been noted with yeasts, bacteria, and even viruses. As a means of sterilization the use of ultrasonic waves has not proved very practical, but they may be significant in the disintegration of cells when it is desirable to minimize the amount of chemical change, or to release enzymes.

CHAPTER 18

Physical Effects Produced by Microorganisms

Microorganisms may produce various physical changes in their environment. They may develop heat and raise the temperature, in some cases they may produce light, they may alter the osmotic pressure, or they may increase or decrease the viscosity of the medium in which they grow.

Heat Production by Microorganisms. All organisms liberate more or less energy in the form of heat as a result of the chemical changes which they bring about. For example, Bayne-Jones and Rhees (1929) determined that each cell of an organism under study growing in a solution produced from 6×10^{-10} to 20×10^{-10} gram calories of heat per hour, depending upon the age of the culture. The amount of heat evolved by some organisms is much greater than that released by others. Organisms producing considerable quantities of heat are said to be *thermogenic* and are usually aerobic. They are widely distributed in nature. They are in evidence in every decaying heap of straw and manure. The heating of a compost heap is due to the presence of optimum conditions for growth of this type of organism. Temperatures as high as 70° or 80° C. may be reached. Frequently the ensilage in a silo undergoes such a process of heating. However, in well-packed ensilage high temperatures are not usually developed except within a few feet of the surface or at points to which air has access. Such heating of silage is due in part to factors other than bacteria, inasmuch as there are oxidizing enzymes present in the living plant cells, which may remain active for a time. The heat evolved in the decay of manure is utilized by the gardener in the maintenance of a suitable temperature in hotbeds.

The heat produced in certain food fermentations (as in the fermentation of cacao beans and pulp) has been claimed to be responsible for hastening certain desirable enzymatic changes in the curing process.

There is evidence that in masses of damp straw or hay the temperature may increase to a point at which spontaneous oxidative changes occur, that is, oxidations not directly brought about by the activity of microorganisms, and the temperature may continue to rise until "spontaneous combustion" occurs. The placing of damp hay in a barn definitely constitutes a fire hazard.

Light Emission. Several terms are used to describe the emission of light. *Luminescence* is the general term which designates that light is being given off. *Phosphorescence* is often used with the same meaning as luminescence when applied to microorganisms; however, it is also applied to the emission of light that is given off only after the exposure of phosphorescent material to light. Probably microorganisms do not exhibit phosphorescence in the latter sense, but some organisms produce pigments that are *fluorescent*. Such pigments absorb light rays of one color, and give off rays of another color, with wavelengths longer than those of the light that was absorbed.

Many microorganisms have been found to be luminescent or *photogenic*. Among them are species of bacteria, fungi, and protozoa. The phenomenon is also known from certain higher animals as well. Most of the luminescent bacteria that have been described are of marine origin. It is not difficult to cultivate them upon artificial media containing preferably a considerable proportion of common salt and sugar. Under these conditions a slant agar culture in a test tube will give off sufficient light so that time may be told by holding the test tube against the face of a watch in a dark room. These organisms are sometimes observed in fish markets or upon decaying fish upon the ocean beach, causing the fish at night to glow with a phosphorescent light. They and various protozoa and minute animals probably account in part, at times, for the phosphorescence of the sea. Bacteria have been identified from the luminescent organs of certain fish and cephalopods. None of them, however, is of great economic importance. The conditions for development are plenty of oxygen and food material, usually the presence of common salt, and a moderate temperature. In addition to the bacteria, some species of fungi are phosphorescent. The mycelium of some of the *Basidiomycetes* (toadstools and mushrooms) is the common cause of luminescence (phosphorescence) in decaying wood and leaves. One of these (*Omphalia flavida*) produces a spot disease upon the leaves of certain tropical plants, particularly on coffee. When growing in the leaf or in a suitable medium the organism is brightly luminescent.

The luminescence of cells is due to the oxidation of certain organic compounds under the influence of a specific enzyme. Harvey has shown that in luminescent copepods the compound *luciferin* is oxidized under the influence of the enzyme *luciferase*. Luminescence of reacting chemical compounds is not unusual; there are many known reactions in organic chemistry in which energy is released in the form of light. It is not certainly known whether the compound oxidized by luminescent bacteria is luciferin.

Changes in the Consistency of the Medium. The changes brought about by microorganisms in the consistency of the medium in which they grow may be divided into two types: those resulting from analytic, and those resulting from synthetic, changes.

Changes Due to Analytic Processes. Many microorganisms are capable of digesting, that is, rendering soluble, solid materials or solid organic substances which may be useful to them as food. The insoluble carbohydrates, such as cellulose and starch, are broken down, converted into simple sugars, or oxidized to relatively simple compounds. A starch suspension, for example, inoculated with an amylolytic organism is rapidly changed from a thick, viscous suspension to one which is relatively clear and limpid. Similar changes are brought about in insoluble proteins and other nitrogenous compounds. Certain organisms when inoculated into a medium solidified by the use of gelatin, hydrolyze this compound, thus destroying its gelatinizing power. Flesh and boiled white of egg or similar substances when acted upon by certain bacteria are rapidly converted into soluble substances, or digested. From the standpoint of the organism, this characteristic is an important asset, inasmuch as it puts insoluble potential food materials into solution and usually in such form that diffusion may take place through the cell wall and plasma membrane, and the organism may utilize the material as food.

Changes Due to Synthetic Processes. Certain organisms may change the consistency of a medium to a considerable degree by formation of products through synthetic action. This is particularly characteristic of those forms which are capable of producing slimes and gums in liquid media. These are usually the result of the secretion of an enormously thickened cell wall or capsule, which rapidly swells and goes into a state of semi-solution or to the direct excretion of gums or mucinlike substances. One may observe the various stages in the production of these materials by the examination of the organisms under the microscope. These slimes or gums may be nitrogenous and mucinlike, or they may be polysaccharides such as dextrans, mannans, and pectins. Organisms producing such compounds may be of considerable economic importance as they are responsible for the production of slimy milk, slimy bread, and gums and slimes in sugar manufacture. It is claimed by certain bacteriologists that many of the so-called vegetable gums produced by higher plants, such as the acacia and astragalus, owe their origin primarily not to direct production by the plant itself, but to the growth of certain microorganisms in the sap exuded.

Changes in Osmotic Pressure. The osmotic pressure of any substance in solution is proportional to the number of molecules (or of molecules and ions) in a given volume of the solution. Any changes in any compound in a nutrient medium which will increase the number of molecules in solution per unit volume will increase at the same time the osmotic pressure. This may be illustrated by the change which occurs in the digestion or saccharification of starch. Starch when boiled passes into a colloidal solution usually somewhat viscous. When inoculated with some organism capable of digesting it, the starch molecules are rapidly broken down into simpler substances, at first into the polysaccharides known as dextrins. The starch in solution has a very low osmotic pressure. The breaking down of the large starch molecule into simpler dextrin molecules increases the pressure somewhat. When these are further broken down into maltose or malt sugar, the osmotic pressure is again increased and when, as may occur, the maltose is broken down into dextrose, the osmotic pressure is once more increased. The analytic action of organisms upon both insoluble and soluble organic molecules, in general, results in increase in the osmotic pressure, while the reverse or synthetic action tends to decrease the osmotic pressure. These facts are of some importance in explaining some of the changes which occur in the tissues of man and animals in certain types of disease. When microorganisms gain entrance to a tissue, destroy the cells, and break them down into simpler soluble and crystalloidal substances, they greatly increase the concentration of solutes and tend to draw the water from the adjoining blood vessels and tissues causing edema and swelling.

CHAPTER 19

Effects of Chemical Environment upon Microorganisms

The chemical elements and compounds present in the environment of microorganisms affect them in several different ways. Some substances are used directly (or after modification) as cell nutrients, either to build cell constituents or to be transformed in such fashion as to yield the energy needed for growth and movement. Study of the nutrition of microorganisms concerns itself primarily with the changes which they bring about in the nutrients. These changes will be considered in detail in later chapters. There are also a number of direct effects of the chemical environment upon cells which are rather indirectly related to nutrition and which will be considered here. These include (1) the effect of the chemical environment upon the movement of microorganisms, (2) the effect upon direction of growth, (3) the effect upon morphology of the cells, and (4) the effect upon rates of growth and of death. Consideration will also be given in later chapters to certain other effects of chemicals such as effects upon rates of metabolism, upon production of adaptive enzymes and upon crop yield of microorganisms in a medium.

THE EFFECT OF CHEMICALS ON MOVEMENT OF MICROORGANISMS

Organisms that have the power of independent movement may have the direction of that motion determined in some measure by the chemical environment. This phenomenon of orientation with respect to a chemical is termed *chemotaxis*. One of the most striking methods of demonstrating this phenomenon is that suggested by Fischer. A capillary tube having a very fine bore is sealed at one end, and the other end is thrust into a solution of peptone or beef extract. Capillarity will cause some of this liquid to enter the tube. The tip of this tube is then placed in a drop of water containing motile bacteria. The direction of their motion may be watched under the microscope. At first they will be seen to be uniformly distributed throughout the drop. Soon, however, the bacteria will cluster about the end of the tube from which the peptone is diffusing. Before long practically all the bacteria will be found at this point and within the tube itself. The cells swim in the direction of increasing concentration of nutrient. (Fig. 19–1A.)

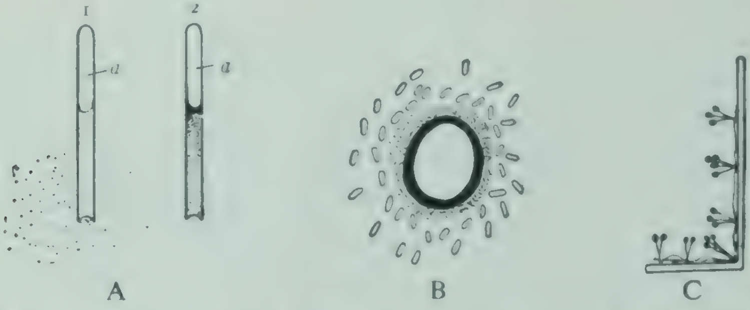

Fig. 19–1. A. *Chemotaxis.* 1, a capillary tube containing an air bubble (a) and meat extract or peptone solution, thrust into a drop of water containing motile bacteria; 2, bacteria have entered the tube and are found in greatest numbers next the air bubble. (Adapted from Fischer.) B. *Aerotaxis.* An air bubble in water under a cover glass surrounded by two rings of organisms, the inner bacterial, the outer protozoan. Each maintains itself in that oxygen concentration which is most suitable. C. *Negative hydrotropism* of mold sporangiophores. *Rhizopus* growing on the bottom and sides of a petri dish. Note that the sporangiophores arise in all instances at right angles to the surface from which they spring or bisect the angle made by these surfaces with each other.

Substances which may serve as food for motile microorganisms do not always attract them. For example, cane sugar, which is an excellent nutrient for many bacteria, will not stimulate chemotaxis. It is probable in nature, however, that chemotaxis is of some use to organisms in finding food. The attraction of motile organisms by a chemical is termed *positive chemotaxis*. The reverse, or repulsion of organisms (*negative chemotaxis*), may be demonstrated by filling the capillary tube with alcohol or an acid. The organisms will be found to shun the vicinity of the tube. Another type of chemotaxis may be demonstrated by placing a drop of stagnant water containing a large number of motile organisms, both bacteria and protozoa, under a cover glass, being sure that several air bubbles are included also. In the course of a few minutes, the organisms will be found to have grouped themselves largely in concentric rings about these air bubbles (Fig. 19–1B). It is evident that these organisms remain in the oxygen concentration which s most suited to their several requirements. This phenomenon is termed *aerotaxis*.

The direction of movement of amoeboid cells is also largely determined by chemotaxis. An amoeba moves in the direction of microorganisms which it may engulf and use as food. Apparently, substances diffusing from the food cells, such as CO_2, influence the direction of motion. Chemotaxis has an important part to play in the destruction of bacteria by the white blood corpuscles in the body, which in certain respects behave like amoebae. Bacteria which attract these white blood cells are engulfed by them and usually destroyed. Many bacteria secrete substances which are thus positively chemotactic. Further, the animal body may be stimulated to the production of substances which are adsorbed specifically by bacteria and make them attractive to the phagocytes.

Chemotaxis may play an important role in the life cycle of motile organisms. For example, the cells of the myxobacters have been described during the vegetative or developmental stages as crawling about over the surface of the nutrient medium, and in the case of the forms which parasitize other bacteria, moving in their direction and digesting them. During the stage of sporulation and fruit body production these movements are quite reversed. The bacteria now tend to crawl together into clumps and to heap up into masses, in some cases stalks are formed, the bacteria pressing to the top where they are transformed into spores. This movement away from the moist substrate may be regarded as an evidence of a negative *hydrotaxis*. A similar phenomenon is also exhibited by the slime molds (*Myxomycetes*). In these forms the component cells are amoeboid instead of rodlike. During the vegetative stage they move about on the substrate, the direction of flow of the mass of fused cells is definitely governed by the chemotactic influence of the location of available food. When the organisms are ready to sporulate, they become negatively hydrotactic and heap up into masses which differentiate into some of the most intricate and beautiful of microscopic plants. The result is that the spores of both slime molds and slime bacteria are produced at some distance from the moist substrate and are in position to be distributed by currents of air when they have become dry.

Effects of Chemicals on Direction of Growth. The direction of growth of nonmotile organisms may be influenced by chemicals. Such a determination of direction is called *chemotropism*. This may be of many types. The influence of moisture upon the direction of growth (*hydrotropism*) is of particular importance. Molds growing upon the surface of nutrient media send their vegetative hyphae into the substratum much as plant roots go down into the soil. The direction of growth in molds is not usually determined by gravity, for in general the mold hyphae grow toward moisture. This may be termed *positive hydrotropism.* On the other hand, some spore-bearing organs of molds, such as conidiophores and sporangiophores, often show marked *negative hydrotropism,* the fertile hypha developing at right angles to a moist surface and producing its spores at some distance from such a surface. The importance of this fact has already been discussed under the heading of mold morphology. Most of the common mold genera, as *Penicillium, Aspergillus, Mucor,* and *Rhizopus,* show this negative hydrotropism very distinctly. (Fig. 19–1C.)

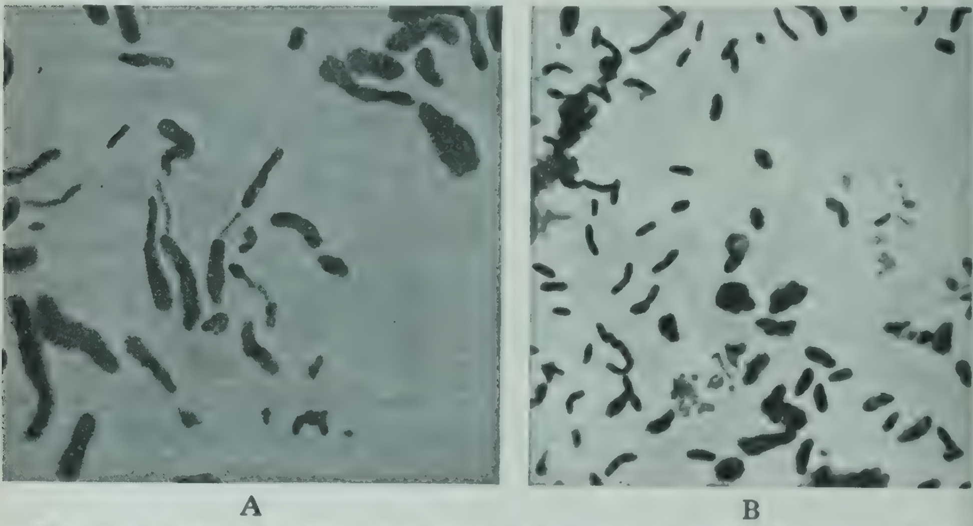

Fig. 19–2. A and B. Variations in shape of certain rodshaped bacteria in old cultures. Cells vary from the relatively slender parent rods to swollen, clubshaped, curved and even spherical.

The byproducts of metabolism of an organism may have a negative chemotropic influence upon other individuals of the same species or other threads of the same mold. An examination of a young culture of mold growing upon some solid media, such as agar or gelatin in a petri dish, will frequently show the mold hyphae radiating from the center in practically straight lines, the new branches rarely crossing the old. This is undoubtedly an example of negative chemotropism, each hyphal thread repelling others near it.

Positive chemotropism is exhibited by many fungus hyphae. Those of certain parasitic forms unerringly find their way through the stomata or the water pores of leaves to the cells from which they can extract nutrients.

Effect of Chemical Environment upon the Morphology of Microorganisms. The chemical environment has a specific effect (*chemomorphosis*) upon the morphology of microorganisms. In fact, the exact form that is assumed by a cell is determined not only by its genetic constitution but also by the chemical and physical characteristics of the environment. These special effects will be discussed in later chapters in relation to the several organisms. Here may be noted some of the principal types of changes that have been observed.

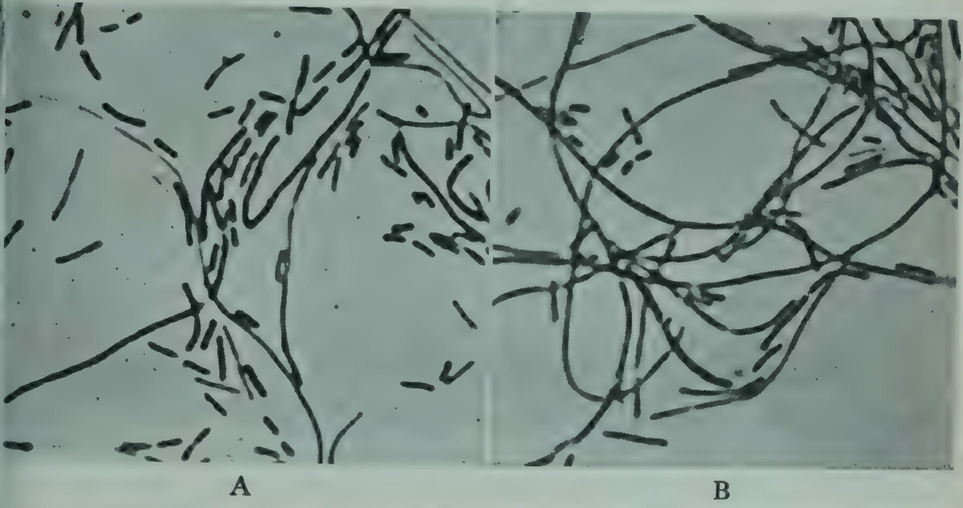

Fig. 19–3. Effect of environment upon morphology. In the presence of dilute penicillin this organism normally growing as short rods develops into greatly elongated filaments.

Microorganisms when grown in a culture medium commonly show changes in the morphology which correspond to the growth phase. Henrici, in particular, has studied the sequence of changes under the general caption of *cytomorphoses*. He noted that during the logarithmic growth phase the cells were in a juvenile stage, followed later by adult and senescent forms. The changes in morphology are intimately related to the changes in the composition of the culture medium produced by the organism.

This fact that the morphology of bacteria may vary with the age of the culture has been taken by bacteriologists rather frequently as an argument for the existence of pleomorphic life cycles resembling somewhat those of the fungi. Prominent among these pleomorphists has been Enderlein, who developed a most fantastic outline of lif cycles for the bacteria. Of this concept Henrici fittingly says:

> Neither the data nor the logic of these new pleomorphists are adequat to convince us that the bacteria possess complex fungoid life cycles. W may sum up the present status of the problem by stating that bacteria i pure culture do show wide variations in the size and form and structur of their cells, but that the nature and significance of these variations ar not known.

Among the variations in morphology that have been observed to b induced by the chemical environment are *size, capsule formation motility, pigment production, sporulation, presence or absence o cell inclusions* such as metachromatic granules, fat globules, glycogen granules.

Yeast and molds are likewise profoundly influenced by their chemical environment. Both stimulation and inhibition of formation of fertile hyphae, conidia, sporangia, and chlamydospores are constantl observed.

Effect of Chemical Environment upon Rates of Growth and Death. Chemicals in the environment of an organism may be inert, they ma stimulate growth, they may inhibit growth, or they may cause rapid destruction of organisms. In other words, the composition of the substrate determines the rates of growth on the one hand and the rates of death of microorganisms on the other.

Effect of Chemicals upon Growth Rates—Stimulants and Nutrients. The chemical constituents of the medium in which an organism is grown may stimulate or modify the growth of microorganisms in an one or more of several ways.

The nutrient may be used more or less directly as a source o energy. A supply of energy is required by every cell; in part, it is utilized in growth, in part it is released as heat; it is sometimes manifest in movement, occasionally in the production of light. This energ is released in all cases by coupled oxidations and reductions. For example, many species of yeast when grown in a medium containin dextrose convert it almost quantitatively into alcohol and carbon dioxide, which are then excreted from the cell. In the transformation energy is released to the cell. The production of alcohol and carbon dioxide from sugar by the yeast cell involves both oxidation and reduction. This process of securing growth energy is sometimes termed *dissimilation*.

Microorganisms which are most frequently studied in the laboratory secure energy by the oxidation of organic carbon compounds; there are, however, many organisms of considerable economic significance which oxidize other substances, such as hydrogen, hydrogen sulfide, sulfur, ammonia, and nitrites.

The nutrient may be used more or less completely in building up cell parts, i.e., *it may be assimilated.* The chemical elements which are necessary for growth of microorganisms are in general the same as those required by other plants and animals. Certainly here are to be included carbon, oxygen, hydrogen, nitrogen, sulfur, phosphorus, and iron. To this list perhaps should be added, at least for some organisms, potassium, sodium, chlorine, calcium, manganese, cobalt and magnesium. These last elements are doubtless *essential to growth,* but it has not been satisfactorily demonstrated in all cases that they are *essential components of the cell itself.* Problems of assimilation will be discussed later.

The substance may act as a growth stimulant without serving directly as an energy source or without being assimilated. Sometimes such growth accelerants function by absorbing or removing from the medium or inactivating substances which are injurious to growth. A chemical which acts as a buffer in preventing an inhibiting change in hydrogen ion concentration will also stimulate growth. Here may also be grouped the long list of hormones, vitamins, and similar growth accelerants which have been shown to be significant (frequently as catalysts) in the metabolism of microorganisms.

Effect of Concentration of Nutrients upon Growth. For each nutrient constituent of a medium there is an optimum concentration at which growth is most rapid. In dilutions sufficiently great, growth may be markedly retarded; increasing the concentration increases the growth rate. For example, with peptone in dilute solution (from 0.0125 to 0.1 per cent), it has been noted that the generation time is inversely proportional to the concentration of peptone used; at higher concentrations (0.4–1.25 per cent), there is little variation in the generation times.

Inhibition of Growth and Destruction of Microorganisms by Chemicals. Chemicals may not only stimulate the growth of an organism, they may also partially or completely inhibit its growth, or they may kill the cell. The terms commonly used with reference to the injurious effects of chemicals upon microorganisms are to be defined. It will be noted that the definitions overlap to some degree.

A *germicide* is any substance which will kill microorganisms. The name is commonly applied to agents destructive to bacteria, particularly to pathogenic forms. A *bactericide* is more specifically any substance that kills bacteria. The term *antiseptic* was originally used to designate any substance which would prevent sepsis, that is, wound and body infection, either by killing outright the microorganisms responsible or by preventing their growth. It is now generally applied to such substances which may be applied to living tissues without injury to the latter. A *disinfectant* defined strictly is any agent which frees from infection; it is usually a substance applied to utensils, buildings, and equipment to free them from pathogenic microorganisms. A *preservative* is any agent which will prevent the growth of microorganisms; the term is used most frequently, though not exclusively, in connection with food preservation. A *deodorant* is any agent which will mask or destroy an offensive odor. A disinfectant may be also a deodorant if it stops production of odors through prevention of growth of the causal microorganisms. A *fumigant* is a gaseous bactericide, fungicide, or insecticide. The suffix, *cide* (to kill), has been generally used in recent years in the coining of terms designating effect of agents on living organisms of many kinds; a *viricide* destroys viruses, a *fungicide*, fungi, an *algicide*, algae, and an *amoebicide*, amoebae.

The noun suffix, *stasis*, connotes quiescence or stoppage. *Bacteriostasis* is stopping the growth of bacteria, but without death of the cells. A *bacteriostatic agent* is one which inhibits growth of bacteria without destroying them. Similarly, *fungistatic* agents stop fungus growth without killing.

Microorganisms may produce in their growth chemical compounds which may be inimical to the further growth of themselves or of other microorganisms. Those which are produced in a medium and make it unfit for further growth of the organism are said to make the medium *stale* and have been termed *staling substances*. Those which either prevent the growth of other microorganisms (are bacteriostatic or fungistatic) or destroy them (are bactericidal) are said to produce *antibiotic* substances. Some of these, as penicillin, streptomycin, and aureomycin, have proved to be particularly useful in combating diseases as the antibiotics are active also when placed in the blood stream. Inasmuch as most antibiotics are products of the growth of microorganisms, they will be treated in detail in a later chapter.

The Course of the Process of Destruction of Microorganisms by
hemicals. What happens when microorganisms (as bacteria) are sub-
ected to the action of a germicide? The answer may be found by
ounting (or estimating) the number of organisms[1] still alive at in-
ervals during the process of cell destruction (disinfection) and deter-
ining the relationships between the number of survivors and the
ime. The results may be plotted, number of survivors against time,
nd a smooth *survivors'* curve drawn. When this is done, in a large
roportion of cases a curve is developed which has the general appear-

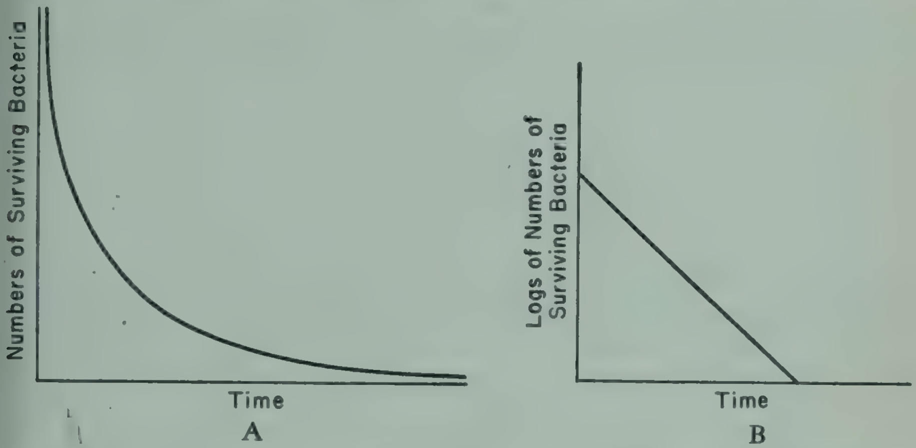

Fig. 19–4. A. A graph of numbers of surviving bacteria plotted against time
n the assumption that equal proportions of bacteria die off in equal periods of
ime. B. A graph of the logarithms of the numbers of surviving bacteria plotted
gainst time, giving a straight line.

nce of that shown in Fig. 19–4A. It will be noted that the numbers of
iving bacteria fall most rapidly at first, then less and less rapidly. The
urve has much the appearance of that secured when one plots the
ecay of radioactive substances against time. The disappearance of
radioactive substance is such that only one half of that present
nitially will remain after a definite period of time, called the half life.
f this applies to bacteria, one half the bacteria present at any instant
ill die in some definite period of time. This may be put in mathe-
atical form.

Let $B =$ number of bacteria at beginning
$b =$ number of survivors after time t
$g =$ length of time for the number of bacteria to be reduced by one half.

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[1] Methods of estimating numbers of living bacteria present have been discussed in
hapter 16.

At the end of the first period, g, the number of bacteria would be halved, or $B/2$. At the end of the second period the survivors would again be halved, becoming $B/2^2$, at the end of n such periods $b = B/2^n$. But the number of periods $n = t/g$, so

$$b = \frac{B}{2^{\frac{t}{g}}} = B2^{-\frac{t}{g}} \tag{1}$$

If the preceding is a true account of the behavior of bacteria in the presence of disinfectants, then this is the equation of the survivors' curve. One may solve the equation for the value of g, the length of time required for the number of bacteria to decrease by one half.

$$g = t\frac{\log 2}{\log B - \log b} \tag{2}$$

Further,

$$\log b = \log B - t\frac{\log 2}{g} \tag{3}$$

Equation 3 is in the form of an equation of a straight line, so if the logarithms of the numbers of survivors are plotted against time, the result should be a straight line, with negative slope of $-\frac{\log 2}{g}$, as shown in Fig. 19–4B.[2]

If microorganisms die in accordance with the principle just developed, the death is said to be logarithmic, or to resemble a first order reaction, or a unimolecular chemical reaction. The test as to whether or not they die as described is easily made; if the logarithms of the

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[2] Another method of derivation of a relationship is to utilize the calculus. Obviously, if in all cases half the surviving bacteria die off in equal intervals, then the rate of death of survivors is constantly proportional to the number of survivors, that is,

$$-\frac{db}{dt} \propto b \quad \text{or} \quad -\frac{db}{dt} = kb \tag{4}$$

in which k is the velocity coefficient of the rate of death, defined by

$$k = -\frac{\frac{db}{dt}}{b} \tag{5}$$

that is, the rate of death per cell (survivor) is a constant. Integrated, this relationship becomes

$$b = Be^{-kt} \tag{6}$$

in which e is the base of natural logarithms.

$$\ln b = \ln B - kt$$

and

$$k = \frac{\ln B - \ln b}{t} = \frac{2.3}{t}(\log B - \log b)$$

numbers of survivors are plotted against time and yield a straight line there is logarithmic death.

In a surprisingly large number of cases survivor curves have been found essentially to be logarithmic. There are many and important exceptions, but the number of agreements has been so large as to lead to the development of many theories regarding the mechanism of disinfection. But it must be kept in mind that all chemical theories relative to reaction rates assume the presence of large numbers of reacting particles (in disinfection, of bacteria) so that there can be an application of the laws of chance with reasonable certainty as to the reliability of prediction. But predictions become uncertain with small numbers. The statistician, by use of actuarial tables, can predict with a high degree of accuracy the number of individuals of any age group that will die during the year. But he cannot predict with any degree of certainty how many of a group of ten will die during the year, and much less predict whether a particular individual will die within the year. From a practical point of view we appreciate the help of the theory in understanding the basic phenomena. But we are most often concerned with the problem of the time required to destroy *all* the organisms, rather than the time required to kill half or some other fraction.

Comparison of Efficiency of Disinfectants. In general, the germicidal power of a disinfectant is measured either by determining the rates of death of organisms in contact with it or by determination of the time required to kill all the microorganisms present.

Theoretically, it would be logical to evaluate disinfectant strength by determining the length of time required to kill one-half or some other definite percentage (sometimes 99.9 per cent is taken) of the organisms initially present. The comparative efficiency of two disinfectants could be determined by the ratio of the times required to kill, say, one-half. The efficiencies would be the reciprocal of the ratio of the values of g determined. Or one might compare directly the determined values of the velocity coefficients of the rates of death. However, methods of counting of survivors at intervals are difficult and tedious and are rarely used. Instead, practical methods of comparing death times have been employed.

Disinfectants act in a great variety of ways, and their germicidal efficiency is much influenced by other elements of the environment. It has therefore proved difficult to develop a wholly satisfactory method of comparative evaluation. For purposes of comparison it has long been customary to accept the germicide phenol (C_6H_5OH) as a standard and to evaluate other germicides by comparison with it. The *phenol coefficient* is determined by comparing the germicidal efficiency of the germicide to be tested with that of phenol under standardized conditions. The conditions of the test have been modified from time to time, the one now usually used is the modification introduced as the result of the studies of the Food and Drug Administration. The following statement briefly outlines the method, but for details a laboratory guide or text on disinfection should be consulted. In making the comparison it is necessary that a standard test organism be chosen. It is customary to use either a special strain of *Salmonella typhosa* or one of *Micrococcus aureus*. A series of test tubes is then prepared, each carrying an appropriate dilution of a standard phenol. Dilutions of 1–80, 1–90, 1–100, and 1–110 are placed, 10 milliliters in each tube, in a water bath kept at 20° C. A similar series of dilutions of the disinfectant to be tested is made, though the exact range of the dilutions will vary with the material under test. If there is no previous knowledge of the range in which the germicide being tested is active, some preliminary tests must ordinarily be made over a wide range of concentrations.

Each of the tubes then receives 0.2 milliliter of the standard culture of the test organism that has been grown for twenty-four hours in broth. At intervals of two and a half minutes a loopful of each dilution is transferred to tubes of sterile broth, such transfers continuing for a total period of fifteen minutes. The broth tubes are incubated for twenty-four hours, and the results tabulated. There should be growth in some of the tubes but not in all. The lowest concentration of phenol and that of the disinfectant under test required to kill the test organisms after exposure of two and a half minutes are recorded. The ratio of the killing concentration of the test material to that of phenol is likewise determined. Finally, the ratio between the concentrations that kill in fifteen minutes is calculated. The average of the two ratios is the phenol coefficient. For example, if phenol kills in two and a half minutes in a concentration of 1–80, and the disinfectant under test in 1–480, the ratio is 6.0. If in fifteen minutes phenol kills in a dilution of 1–100 and the test material 1–800, the ratio is 8.0, and the phenol coefficient is the average of the two ratios or 7.0.

Many other methods of testing and comparing disinfectants have been developed for particular purposes or as related to special groups of organisms. For example, the evaluation of disinfectants useful on sporulating bacteria is quite a different problem. The relative toxicity of a chemical compound to tissue cells of the body in culture and its germicidal action upon certain bacteria may be of great practical importance.

Characteristics of an Ideal Germicide. No germicide can be ideal in all its characteristics. However, it is helpful to list the characteristics of such an ideal disinfectant, for to the extent that any disinfectant measures up to those of the ideal, it should be important. Here, then, are some of the characteristics to be checked.

1. *High Germicidal Power*. The ideal disinfectant should possess high germicidal power. It has been seen that this is usually measured by determination of the phenol coefficient.

Further, the germicidal activity should not be too narrowly selective; microorganisms of many types, particularly pathogenic bacteria, should be destroyed.

2. *Stability*. A disinfectant to be most useful usually should be relatively stable in the presence of organic matter. Some of the most powerful of the disinfectants combine with organic matter, forming insoluble compounds and pass out of solution almost completely. The strength of the disinfectant may thereby be decreased rapidly to a point where it no longer destroys microorganisms. In some cases lack of stability may be desirable rather than undesirable, if, for example, the disinfectant is at first very active and later loses its efficacy. Water supplies of cities are often treated with gaseous chlorine, a powerful germicide. It rapidly combines with other compounds present in the water and becomes innocuous. The water is then potable. In short, the chlorine first sterilizes the water and then disappears.

3. *Homogeneity*. Disinfectants should be homogeneous in composition. Substances which may be secured pure as in crystalline form, such as mercuric chloride, are ideal from this point of view. Many of the commercial disinfectants, particularly those prepared from coal tars, may vary considerably in their composition from time to time and consequently in their germicidal value.

4. *Solubility*. The ideal disinfectant is one which will dissolve in all proportions in water. If it has a very high germicidal power, high solubility is not so important.

5. *Non-Toxic to Higher Life*. An ideal disinfectant would be one which is non-poisonous to man and animals. Obviously disinfectants which will kill one kind of cell and not injure another are difficult to find. Most of the valuable disinfectants are more or less injurious to

tissues. Certain disinfectants, however, may be injected into the blood, exerting a more harmful influence upon microorganisms than upon tissues of the body, destroying the former without seriously injuring the latter. Such, for example, are certain arsenic compounds used in the treatment of syphilis, and the sulfa compounds used in the treatment of many diseases. A whole new array of compounds relatively non-toxic to animals and man are the antibiotics produced by various microorganisms and even higher plants, to be discussed in Chapter 23.

6. *Non-Corrosive and Non-Bleaching.* The ideal disinfectant should not corrode metals or bleach fabrics.

7. *Power of Penetration.* A disinfectant should penetrate readily.

8. *Cheapness.* An ideal disinfectant should be readily secured and relatively inexpensive.

9. *Deodorizing Power.* A disinfectant is the more acceptable if it combines with or absorbs odors, i.e., serves as a deodorant.

10. *Cleansing Power.* The ability to remove dirt and grease is highly desirable. It should wet surfaces readily and act as a detergent.

How Germicides Act. Germicides destroy microorganisms in many ways, some well understood and others obscure. Several of the more important types of action will be noted.

Some disinfectants are useful because of their powerful action as *oxidizing agents.* They destroy more or less completely the organic matter of the cell. For example, potassium permanganate acts in this manner. Other germicides cause more or less rapid digestion, *hydrolysis* or solution of the cells, thus destroying them. A strong acid or a strong alkali may produce this change. Other chemicals apparently alter the *permeability* of the cell membranes so that they cannot function satisfactorily. In other cases there is a direct *chemical union* with certain cell constituents. The cell is killed as a result, the protoplasm in some cases being coagulated or denaturated. Such changes are produced by formaldehyde and by mercuric chloride. The death of the cell may be occasioned by a mechanical disruption or injury, such as a plasmoptysis or a plasmolysis due to differences in osmotic pressure. This type of action is probably, in part at least, the explanation of the preservative action of strong brines and syrups. In some cases a chemical may prove injurious to the cells of microorganisms because primarily it accelerates or stimulates the activity of some other substance which is primarily germicidal. The addition of common salt, for example, to a solution increases the germicidal activity of phenol because it tends to increase the amount of phenol taken up by the cell. Some substances block the activity of essential enzymes by combining with them and preventing them from uniting with and transforming compounds normally metabolized and essential to growth. For example, the optical isomers of certain essential amino acids may prevent the utilization of the latter.

Some disinfectants, particularly the salts of the heavy metals, are most efficient in solutions in which they are strongly ionized. For example, mercuric chloride dissolved in absolute alcohol in which it does not ionize is less efficient than when in solution in water, in which it does ionize. Other substances present may markedly modify the action of the disinfectant. The amount of ionization, for example, may be wholly changed by the addition of salts.

A phenomenon sometimes of significance is that of *ion antagonism*. For example, a solution of sodium chloride is injurious to the bacterium of meningitis, but the effect may be neutralized by the addition of suitable amounts of potassium or calcium salts.

Disinfectants, antiseptics, and preservatives in common use may, for convenience, be grouped under the following heads: salts of the heavy metals, the arsenicals, the halogens and their compounds, alkalies and mineral acids, organic compounds and dyes, and antibiotics.

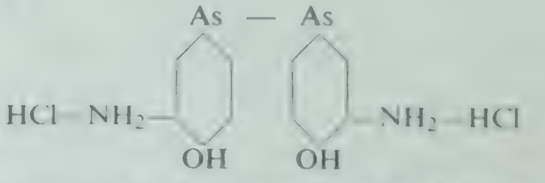
Salts of the Heavy Metals. The soluble salts of the heavy metals—mercury, silver, copper, and iron—are more or less efficient as disinfectants.

Mercuric chloride has been frequently used as a disinfectant, and under the right conditions it is efficient. It combines with protoplasm to form an albuminate of mercury, an insoluble compound. It must be used in considerable excess when disinfection of solutions containing quantities of organic matter is desired (feces, for example), because of formation of insoluble compounds. An impervious coating may be formed over the surface of solid particles and may protect the bacteria in the interior from injury. Mercuric chloride in high dilutions acts as an antiseptic, preventing growth of organisms in a solution containing one part in 100,000 or even 1,000,000. It is usually employed as a disinfectant in a strength of 1–500 or 1–1000. Spores of most bacteria are killed in water by this concentration in an hour or less; vegetative cells are destroyed within a few minutes. This compound may be purchased mixed with other substances in tablet form, the other ingredients being such as to lessen the formation of insoluble precipitates, thus rendering the solution more effective. Mercuric chloride is so toxic to animals and man, however, that its use in the household is not advisable. The germicidal effect of mercury is more or less completely neutralized by the presence of compounds containing —SH. Its germicidal action is due to combination in the cell with essential compounds, such as glutathione, which contain —SH. Bacteria apparently dead as a result of contact with mercury may be rendered again viable by treatment with —SH compounds. It also corrodes metals with which its solutions come into contact. Many organic compounds of mercury such as mercurochrome and methiolate have been introduced as having low toxicities for animal tissues but with relatively high phenol coefficients. *Mercuric iodide* may be used as is the chloride and is even more efficient.

Silver nitrate is relatively effective as a disinfectant and is used therapeutically, particularly to kill bacteria on body surfaces. Colloidal suspensions of silver are also used to destroy organisms on mucous membranes.

Copper is one of the most efficient of chemicals as an algicide, particularly in water reservoirs. Such filamentous forms as *Spirogyra* are killed by even as low a concentration as 1 part per million of copper sulfate. Contaminated water in city supplies has been treated, but for this purpose it has been superseded in most instances by chlorine and its compounds. It has proved useful in some cases in the sanitary control of swimming pools. It is an essential ingredient in many fungicides and in combination with lime (as Bordeaux mixture) is commonly used in sprays to prevent plant diseases.

Arsenic and Antimony Compounds. Arsenic has been found useful in the destruction of certain microorganisms particularly when it is in the form of an organic non-ionizable compound. The first noteworthy discovery was that of Ehrlich, who synthesized an organic arsenical marketed under the trade name of salvarsan and in America as arsphenamine. Its dihydrochloride has the following structural formula:



He found that it was relatively non-toxic when injected into the blood stream, but actively killed the treponemata of the disease syphilis. Numerous other arsenic compounds were later introduced, several of considerable significance in treatment of diseases caused by spirochetes and amoebae. The introduction of these compounds and their proved value as specifics in the cure of certain diseases constituted a great stimulus to the search for other compounds which could also be rated as specifics, a search which has been partially successful, as witness the discovery and use of the so-called sulfa drugs.

Organic compounds containing *antimony* have been found useful in the treatment of certain tropical diseases produced by animal parasites. They are relatively non-bactericidal.

Halogens. All the halogens (chlorine, bromine, iodine, fluorine) are actively germicidal in the free state, as are also some of their compounds.

Free Chlorine is one of the most widely used of germicides. It is added to the water supply in many cities where there is danger of pollution. It has the advantage of being very active as a disinfectant, but it soon disappears as free chlorine, combining with other substances present. It is used in concentrations of one-half or less to several parts per million. It has also found extensive use in the disinfection of water in swimming pools, and in sterilization of sewage effluents. The *hypochlorites,* calcium hypochlorite ($CaClOCl$) in particular, may also be utilized to prevent infection through contaminated city water supplies, but it is usually replaced by free chlorine. Under various trade names hypochlorites are sold extensively on the market for the sterilization of dairy utensils, the washing of glass beverage containers, dishes, and containers used in the dispensing of food. An alkaline solution of hypochlorite has been widely used in wound disinfections in medicine; it has been named Dakin solution, after the man who developed it. A strong alkaline solution of hypochlorite under the trade name of antiformin has also been used as a disinfectant. The *chloramines* are compounds in which the hydrogens of amino ($-NH_2$) or imino ($=NH$) groups are substituted by chlorine, giving $-NCl_2$ or $=NCl$. For example, dichloramine T is $CH_3{\cdot}C_6H_4{\cdot}SO_2{\cdot}NCl_2$. *Chlorides* are not generally efficient in low concentration, but when present in excess, they exert a decided preservative action. Few organisms can develop in a brine containing more than 20 per cent of common salt. Advantage is taken of this fact in the use of salt and brine in pickling and preserving foods.

Fluorine, as sodium fluoride, is sometimes used to arrest the growth of organisms when it is desired to study the activity of enzymes present. Certain enzymes act readily even in a one per cent solution of sodium fluoride, while the organisms present are inhibited in growth or completely destroyed, and other enzymes are inactivated.

Iodine and its compounds are commonly employed as disinfectants, usually in alcoholic solution. The standard tincture of iodine contains 2 to 7 per cent iodine and 5 per cent potassium iodide. It is often used in attempted disinfection of wounds.

Acids and Alkalies. These are usually significant as a result of the concentration of hydrogen and hydroxyl ions which they dissociate, in other words, upon the changes in pH they induce.

Acids may be effective as germicides or preservatives due to the hydrogen ions dissociated and the direct effect of such ions upon the microorganisms; with some acids the cation may also be significant, as also the undissociated acid molecules. The latter are particularly significant with certain organic acids. Acetic acid owes much of its preservative action to the undissociated acid rather than to the hydrogen ions. With many (not all) inorganic acids the hydrogen ions are most significant.

Some bacteria are surprisingly resistant to high concentrations of hydrogen ions, particularly certain forms which themselves produce acid. *Thiobacillus thiooxidans*, which oxidizes free sulfur to sulfuric acid, may produce a tenth normal acid solution, with a pH 1.

Sulfurous acid is one of the most commonly used of disinfectants, particularly in fumigation. The sulfur dioxide is prepared by burning sulfur. When dry, it is of little value, but in the presence of moisture, as in vapor-saturated air, it is quite efficient. Under these conditions sulfurous acid is formed. It has the disadvantage of being an active bleaching agent. It will remove the color from carpets, curtains, wallpaper, etc., when moist. It is still commonly used in the fumigation of ship holds, where it is desired not only to destroy microorganisms of all kinds, but also rats, insects, and other vermin. About four pounds of sulfur should be burned to every thousand cubic feet of air space. Water should be boiled or steam should be let into the room at the same time. As a household fumigant it has been almost entirely replaced by formaldehyde.

Hydrogen ions frequently greatly increase the effectiveness of other germicidal agents. High hydroxyl ion concentration may also markedly accelerate bacterial death rates.

Lime, either calcium oxide or hydroxide, is frequently used as a disinfectant in privy vaults and in the form of whitewash in outbuildings.

It is efficient when fresh, but on exposure to the air is gradually changed into calcium carbonate which has little or no germicidal action. Whitewash is an excellent aid to the preservation of cleanliness, but it should not be relied upon as a disinfectant.

Alkaline compounds such as sodium carbonate, sodium hydroxide, and sodium phosphate are the principal constituents of the washing powders commonly employed in creameries and other establishments where many bottles are washed. If sufficiently concentrated, these are effective disinfectants as well as detergents.

Organic Compounds. The most commonly used organic germicides and preservatives are formaldehyde, chloroform, iodoform, alcohol, organic acids such as acetic and lactic, phenol and its derivatives, the cresols, benzoic acid, salicylic acid, certain of the aniline dyes, toluol, quaternary ammonium compounds, and a few of the essential oils such as oils of peppermint, clove, thyme, and cinnamon.

In addition, there are numerous organic compounds that are significant in therapy, as quinine, optochin, the sulfa drugs, and the antibiotics.

Formaldehyde (HCHO) is a gas readily soluble in water and commonly sold as formalin, a 40 per cent or saturated solution in water. It may also be secured in the form of one of its polymers called "paraformaldehyde" and "trioxymethylene." Both of these polymers are readily converted into formaldehyde by heat. Formaldehyde may be prepared also by partial oxidation of methyl alcohol which occurs when it is passed through finely divided platinum raised to red heat. Formaldehyde is at present more commonly used in fumigation than is any other gas. It does not injure the texture of fabrics and very rarely modifies or changes their colors in the least. It can therefore be used for the disinfection of furnished rooms. It is active only in the presence of moisture, and during fumigation a room should have its atmosphere as nearly saturated as practicable. Formaldehyde destroys organisms by uniting chemically with the protoplasm. About 1 per cent by volume of the gas is necessary for efficient disinfection of rooms. Many methods have been devised for the production of formaldehyde in quantities sufficient for fumigation. The most common method used is that of heating formalin directly over a flame in a suitable vessel. The heat at first converts most of the formaldehyde into paraformaldehyde, but as the water evaporates this is broken up and given off as formaldehyde gas. By this method a sufficient quantity of moisture is always introduced. Much the same result can be obtained by placing potassium permanganate crystals in an earthen or wooden vessel and pouring the solution of formalin over them. The rapid oxidation of a part of the formaldehyde by the permanganate results in the production of a sufficient amount of heat to vaporize most of the remainder. This method may be used conveniently where it would be unwise to introduce a fire into a closed room. Many types of apparatus are also on the market for the production of formaldehyde from its isomers. In addition to the use of formaldehyde as a fumigant, it has sometimes been utilized as a preservative in food, as in milk. Its use for this purpose is now generally forbidden by law.

Chloroform ($CHCl_3$) when pure does not readily destroy microorganisms. Its most important use is as a preservative. When added to certain materials such as blood serum it will prevent or retard the growth of bacteria. It is also used as an antiseptic in the investigation of changes brought about by enzymes in order to prevent the growth of organisms that might obscure or modify the results of enzyme action. It is not very efficient inasmuch as some organisms can grow readily in media lying above chloroform, as in a tube of bouillon having a layer of chloroform at the bottom.

Iodoform (CHI_3) is commonly used as an antiseptic by physicians. It has but little direct action upon microorganisms and is not itself actively an antiseptic or disinfectant. When in contact with organic compounds it may free some iodine, which has a distinct germicidal action.

Certain of the organic acids, particularly *lactic* ($CH_3{\cdot}CHOH{\cdot}COOH$) and *acetic* ($CH_3{\cdot}COOH$) are among the most commonly used preservatives. The decomposition of milk proteins is prevented by the formation of lactic acid by certain bacteria, and it is only after this acid has been oxidized to carbon dioxide and water by certain molds that further changes in the milk take place and the protein compounds are broken down. Lactic acid is also formed in sauerkraut and various types of pickles (such as dill pickles) by the fermentation of the sugars present. It accumulates in sufficient quantities to inhibit more or less completely the growth of putrefactive organisms. Acetic acid, the active constituent of vinegar, is formed usually by the oxidation of alcohols by bacteria. It inhibits the growth of many microorganisms and is frequently used as a preservative, as in pickled meats, vegetables, and fruits.

Ethyl alcohol (ethanol) unmixed with water has very little disinfecting value; in fact, absolute alcohol apparently abstracts water from organisms without denaturation of the cell proteins. In some cases both sporulating and non-sporulating bacteria may be kept viable in alcohol for considerable periods of time, even for years. When thus dehydrated, the cells are also much less sensitive to high temperatures. *Micrococcus aureus* in absolute alcohol has been subjected to temperatures as high as 120° C. for an hour without killing all the cells. However, 70 per cent alcohol is markedly bactericidal and in this concentration apparently denaturates (coagulates) the bacterial protoplasm. The higher alcohols such as propyl, butyl, and amyl have real bactericidal powers.

Phenol (C_6H_5OH) or carbolic acid was one of the first widely accepted disinfectants. It is commonly used in 5 per cent solution, which will destroy non-spore-bearing microorganisms very quickly. Some bacterial spores, however, can resist its action for a long time. The addition of 0.5 per cent of hydrochloric acid increases its effectiveness considerably. All the common pathogenic bacteria, with the exception of those producing spores, are destroyed within a few minutes by such a mixture. It is particularly valuable because it does not form insoluble precipitates with albuminous substances. It is the *standard* disinfectant, that is, it is the compound with which other germicides are compared in determining their disinfecting value, i.e., the phenol coefficient. It should not be inferred from this fact that it is an ideal, or even the best, disinfectant. Many substituted phenols have high germicidal efficiency. Among these is *cresol* or *methyl phenol* ($CH_3{\cdot}C_6H_4{\cdot}OH$), one of the chief constituents of many of the proprietary disinfecting compounds upon the market. Three types are known to the chemist, the meta-, the ortho-, and the paracresol. A mixture of these three is called *tricresol*. A saturated solution in water contains about 2½ per cent. In this concentration it is two or three times as efficient as phenol. The cresols are sometimes mixed with strong acids or alkalies to increase their solubility. Such mixtures are sold under various trade names and in most cases are efficient and reliable.

Benzoic acid ($C_6H_5{\cdot}COOH$) and *sodium benzoate* ($C_6H_5{\cdot}COONa$), *salicylic acid* ($C_6H_4{\cdot}OH{\cdot}COOH$), and *sodium salicylate* ($C_6H_4{\cdot}OH{\cdot}COONa$) are commonly used as preservatives in foods, usually in quantities of 0.2 per cent or less. In the United States the use of salicylic acid or salicylates in food is usually forbidden, while the use of benzoate is permitted, provided its presence and amount is stated.

Miscellaneous Compounds. Essential oils such as *thymol* and *eucalyptol* exert a marked antiseptic or even disinfectant action. Others, such as oils of peppermint, cloves, and cinnamon, are somewhat less effective. The addition of spices and their essential oils undoubtedly plays a part in the preservation of certain food products.

Hydrogen peroxide (H_2O_2) will inhibit bacterial growth in dilutions as high as 1–20,000. Its action is probably similar to ozone (O_3).

Potassium permanganate is an active oxidizing agent in solution, destroying most organic matter with which it comes in contact.

Boric acid is sometimes used in saturated solution as an antiseptic, particularly in treatment of bacterial infections of mucous surfaces.

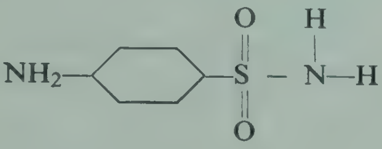
Quinine and the related compounds may be cited as an example of the group of alkaloids which have considerable therapeutic value, particularly in the treatment of protozoan infections as malaria in man.

Bile and Bile Salts (salts of *Taurocholic Acid*). These have been used for two purposes. Certain bacteria dissolve when bile is added to a suspension of the cells. This characteristic is used as a routine test for the differentiation of the organism frequently causing pneumonia (*Diplococcus pneumoniae*) which is bile soluble from the morphologically somewhat similar species of *Streptococcus*. Bile is also used as an ingredient of certain media. In suitable concentrations it will inhibit the growth of many kinds of bacteria, such as gram-positive forms and not interfere with the growth of others, such as certain members of the colon typhoid group of bacteria. Bile is therefore used both in enrichment and selective media.

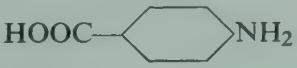
Detergents. These are compounds which have a cleansing action by changing surface tension and by increasing the wetting power of water in which they are dissolved. The classic example, of course, is soap, usually the sodium or potassium salts of the higher fatty acids. Ordinary soap has relatively low germicidal activity, although it removes organisms from surfaces due to its detergent action. In recent years there has been a tremendous expansion in research on the preparation of new synthetic detergents. They have been described by the hundreds, and scores are marketed. Many of them possess characteristics superior to those of soaps in that they may be used in acid or alkaline solutions and in hard waters. Some of them are ionizable with the detergent property resident in some cases in the anion, in others in the cation. Some detergents do not ionize. Many of these detergents actively inhibit the growth of bacteria.

Quaternary Ammonium Compounds. These are closely related to the synthetic detergents. They are characterized as nitrogenous compounds with one halogen atom (usually chlorine) and four organic radicals united to the nitrogen. Scores have been prepared, some showing relatively high germicidal efficiency. They are finding increasing use for many purposes. Certain viruses are selectively destroyed, as are also certain bacteria.

Sulfonamides. Certain snythetic drugs which contain the sulfanilamide radical



with one of the amino hydrogen atoms substituted by various organic radicals have proved to be of value in the treatment of some infectious diseases caused by microorganisms. These have been produced in great variety, and the use of some of them has been spectacularly successful. In general, these compounds are not highly bactericidal, they tend to inhibit growth rather than to kill, they are bacteriostatic in action. Apparently, one of these compounds may hold an invading organism in check, and the body then disposes of it. Of special interest is the fact that a hint as to the mode of action of certain of these drugs is to be found in the fact that their efficacy can be overcome by presence of sufficient amounts of the compound para-amino benzoic acid



(frequently abbreviated PAB in the literature), which is essential in the metabolism of some kinds of microorganisms. It will be noted that PAB structurally somewhat resembles the sulfinamides. Apparently the latter block the utilization of PAB, probably by combining irreversibly wtih the enzyme which normally metabolizes the PAB.

Aerosols. In addition to gases such as formaldehyde and sulfur dioxide, some use has been made of mists of certain disinfectants to destroy microorganisms in air. Such suspensions of fine droplets (preferably as small as 1 to 2 μ in diameter) have been termed aerosols. Of the many compounds tested, certain of the glycols, particularly propylene glycol ($CH_3 \cdot CHOH \cdot CH_2OH$) have proved most useful. A concentration as fine mist of 1 gram of propylene glycol has been found promptly to kill most bacteria (spores excepted) that may be present in as much as 2000 to 4000 liters of air. The glycol is most efficient in air below 80° F. and with a relative humidity between 45 and 70. The material is odorless and apparently non-toxic. Triethylene glycol resembles propylene glycol in its action.

Dyes. Dyes are used in the bacteriological laboratory not only to stain microorganisms and as indicators, but in some cases also because they constitute excellent germicides or fungicides, or are bacteriostatic or fungistatic. For example, suitable concentrations of crystal violet will inhibit the growth of most gram-positive bacteria (acid-fast bacteria excepted) without interfering materially with the development of most gram-negative forms. Certain dyes are therefore used in enrichment and differential media. For example, in peptone water, malachite green in a concentration 1–10,000,000 destroyed the gram-positive *Micrococcus aureus* while a concentration of 1–1000 was required to kill *Escherichia coli*, a gram-negative form.

CHAPTER 20

Cycles of the Elements in Nature

The purpose of this chapter on cycles of the elements in nature is to outline briefly the nature of certain of the changes induced and to note the part played by microorganisms in inducing these changes. The elements requiring emphasis are nitrogen, carbon, and sulfur. The specific changes brought about in chemical compounds will be discussed in detail in succeeding chapters.

The Cycle of Nitrogen in Nature. Nitrogen exists in nature in three principal forms: free in the air as a gas, in inorganic compounds such as ammonia, nitrites, and nitrates, and in organic compounds such as amino acids and proteins. Microorganisms are important in changing nitrogen from one form or combination to another; in fact, without their activity it would be impossible for higher animals and plants to exist.

It is most convenient to start with complex organic nitrogenous compounds in a study of the nitrogen cycle. The changes to be considered are *ammonification, nitrification, nitrogen assimilation, denitrification,* and *nitrogen fixation.*

Ammonification. The conversion by microorganisms of complex nitrogenous compounds into simpler forms and ultimately into ammonia by the process of hydrolysis is termed *ammonification.* This occurs in several distinct stages. The proteins of plants and animals are hydrolyzed into somewhat simpler compounds, the *proteoses,* and then into *peptones;* this process may be termed *peptonization.* These peptones in turn are broken down by various organisms with the formation of polypeptides and *amino acids* primarily and a considerable number of secondary products. The amino acids are still further decomposed with the production of *ammonia.* This whole process of successive cleavages of proteins is sometimes termed *proteolysis.* One of the principal nitrogenous waste products of the dissimilation of proteins in the animal body is urea. This is actively transformed by soil bacteria into ammonium carbonate. All organic nitrogenous compounds may be ultimately reduced to ammonia by the process of ammonification. This is of very great economic importance to agriculture, as nitrogen in this form in soils is readily available to higher plants.

The various steps in ammonification may be brought about by different organisms. A few species can attack native proteins; many, however, can utilize and change only the peptones, the peptides, and amino acids. These will be considered in some detail later. The organisms changing urea to ammonium carbonate constitute a relatively distinct group.

Nitrification. Certain bacteria, particularly those belonging to the genus *Nitrosomonas,* are able to oxidize ammonia to nitrous acid. They are common in the soil. They are autotrophic forms which secure their energy for growth and for chemosynthesis by this oxidation. The nitrous acid is normally at once neutralized by the bases of the soil, thus forming nitrites. These nitrites soon undergo the next change, oxidation to *nitrates* by other species of soil bacteria (of the genus *Nitrobacter*). The nitrates, and to a less degree the ammonia, constitute the most commonly used sources of nitrogen for higher plants. No nitrogenous manure is effective in increasing crop yield that is not capable of being ammonified and nitrified.

Nitrogen Assimilation. The discussion of the nitrogen cycle is not complete without a consideration of nitrogen assimilation, even though this is in large part a function performed in nature by the higher plants. The nitrates, and to a less degree the ammonia, produced by bacterial activity in the soil, are taken up through the roots and built up into protoplasm and complex proteins. These may decay or they may be eaten by animals, but ultimately they are decomposed by microorganisms. This alternation of synthesis of proteins by higher plants and disintegration by microorganisms constitutes a major part of the nitrogen cycle.

Denitrification. Nitrates in the absence of oxygen and in the presence of organic matter may be reduced to nitrites by certain microorganisms, and these nitrites further decomposed with evolution of free nitrogen. Under these conditions microorganisms utilize the nitrate or nitrite as hydrogen acceptors, removing the oxygen. Some species of bacteria, for example, will live under anaerobic conditions if nitrates are present; otherwise they are aerobic. This fact is of some significance in agriculture in explaining loss of fertility in water-logged soils.

Nitrogen Fixation. It is evident that if gaseous nitrogen is lost from the cycle as a result of denitrification, there must be some method whereby it can be again fixed or combined. Many species among the bacteria and molds are known to possess this power.

Certain of the higher plants have fungi which live upon or in their roots and which in a few cases probably take up the atmospheric nitrogen. Among these are the alders, Russian olives, and many other trees, the heathers, the orchids, and many plants living in peat bogs and acid soils. These symbiotic combinations of roots and organisms are termed *mycorrhizas.*

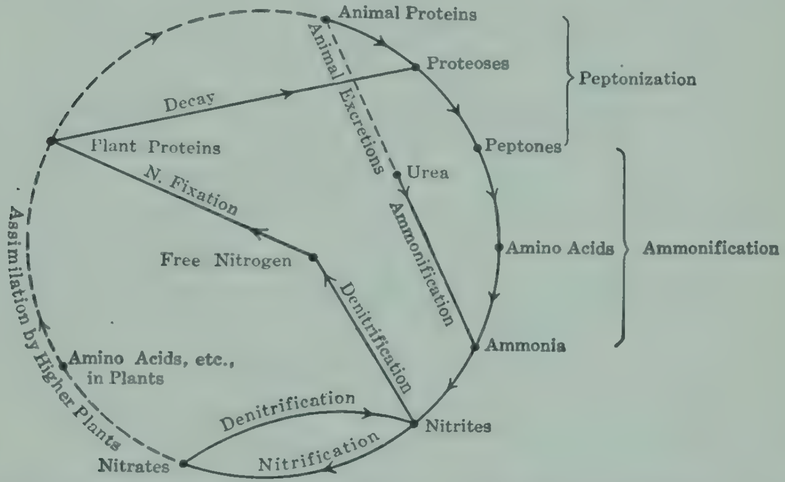


Fig. 20–1. Diagrammatic representation of the cycle of nitrogen in nature. The changes represented by solid lines may be produced by microorganisms; those shown by use of dashes are produced by higher plants or animals.

Various species belonging to the bacterial genus *Rhizobium* (as *Rhizobium leguminosarum*) induce production of nodules on the roots of many leguminous plants such as the bean, pea, clover, and alfalfa. These nodules are made up of several kinds of cells, some of them tightly packed with bacteria. In some way the bacteria and the plant cells are together able to take nitrogen from the air and directly or indirectly transfer it in part to the host plant. Legumes, unlike most other plants, therefore, can grow in soil relatively deficient in nitrogen, provided the roots are supplied with nodules. The efficiency of legumes in increasing the fertility of the soil is due to this fixation of nitrogen.

There are also free-living soil bacteria which can take up nitrogen from the air. These belong to two groups, anaerobes and aerobes. Some spore-bearing soil bacilli (clostridia) in the presence of proper food, such as certain carbohydrates, can fix some nitrogen under anaerobic conditions. These forms are probably not very important in most soils. Much much important are the aerobic organisms belonging to the genus *Azotobacter*. These bacteria secure their energy by the oxidation of carbohydrates.

Summary. These changes of nitrogen are summarized in the accompanying diagram (Fig. 20–1). The direction of changes is indicated by arrows, the changes produced by microorganisms are shown by solid lines, and other changes by broken lines.

The student should note that comprehension of the nitrogen cycle and of the various changes included is useful as a basis for understanding many of the changes of economic significance produced by bacteria. A study of soil bacteriology is in large part a consideration of the changes produced in nitrogenous compounds by microorganisms. The ripening of certain foods includes consideration of proteolysis and ammonification; food spoilage is frequently concerned with changes in proteins and their products of hydrolysis. In problems of sewage disposal and water purification the processes of ammonification and nitrification are of great practical significance.

Carbon Cycle. The cycle of carbon in nature as affected by microorganisms is somewhat simpler than that of nitrogen. It may be best understood by emphasizing two fundamental facts: first, that most living organisms, plants and animals alike, are continuously developing carbon dioxide, which usually goes into solution or is dissipated in the atmosphere; second, that some cells can synthesize organic compounds, principally carbohydrates and fats, from carbon dioxide taken from the environment. The conditions for these antithetic transformations should be understood. Most active cells are constantly breaking down carbon compounds with production of CO_2; some can also build up complex carbon compounds from CO_2 as a carbon source. All plants containing chlorophyll (leaf green) produce starch or other organic compounds from water and CO_2, gaining the energy necessary for the reduction of the CO_2 by means of the absorption of sunlight. A few bacteria (those containing bacteriopurpurin or bacteriochlorin) are also capable of using light for this purpose. Some forms oxidize ammonia to nitrites, nitrites to nitrates, hydrogen sulfide to sulfur, or sulfur to sulfuric acid and utilize the energy thus secured in part in assimilating CO_2 and building up food materials.

It is evident that the carbon cycle consists of the alternate building up of carbon into organic compounds and their subsequent disintegration with ultimate oxidation of the carbon to carbon dioxide (Fig. 20–2). The various stages in decomposition and the products of action of organisms on carbon compounds will be considered in a subsequent chapter. Many of these changes are of great economic importance.

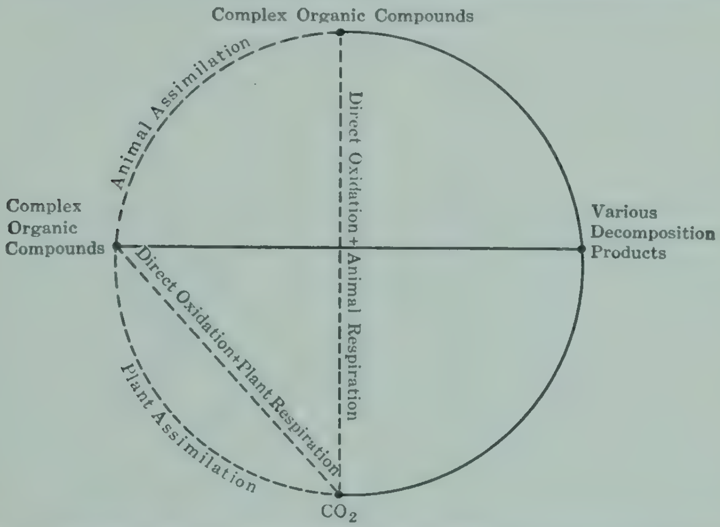

Complex Organic Compounds
Animal Assimilation
Direct Oxidation + Animal Respiration
Complex Organic Compounds
Various Decomposition Products
Direct Oxidation + Plant Respiration
Plant Assimilation
CO_2

Fig. 20–2. Carbon cycle. Changes that are produced by microorganisms are shown by solid lines, others by dash lines.

Sulfur Cycle. Most proteins contain sulfur and upon hydrolysis yield certain sulfur amino acids, as cystine. The compounds are readily attacked by microorganisms, frequently with the transformation of the sulfur into hydrogen sulfide. In some cases, as with the mold *Microsporum gypseum,* the sulfur is oxidized and appears as sulfates.

Wherever hydrogen sulfide is formed, whether originating from decomposition of organic matter or present in natural waters as sulfur springs, under aerobic conditions it is rapidly oxidized by bacteria. In some cases the hydrogen sulfide may be oxidized to free sulfur, in others to the various sulfur acids and thiosulfates. Reduction of sulfates with formation of hydrogen sulfide occurs in the presence of organic matter under anaerobic conditions. The sewages of some cities, for example, are particularly offensive because the city water supply contains sulfates in considerable quantity, the bacteria causing decomposition of the organic matter of the sewage reduce the sulfates, and the sulfide is formed. The economic significance of certain of these changes will be noted later.

Other Elemental Cycles. Somewhat similar cycles of elements in nature showing the influence of microorganisms upon the changes may be established, notably for phosphorus, potassium, and calcium.

CHAPTER 21

Basic Mechanisms of Chemical Changes Produced by Microorganisms

Microorganisms, particularly the bacteria, yeasts, and molds, are important in bringing about a great many chemical changes. In the course of the development of our knowledge of these changes several terms have come to be used, in some cases with gradual modification in meaning with increased understanding. These names are so commonly encountered that they require definition; however, they are sometimes used so loosely, or with such a variety of meanings, that an adequate definition is in some cases difficult or impossible. They can best be understood by some discussion of their history. Among these terms are *respiration, fermentation, putrefaction, decay, digestion, dissimilation,* and *ferment.*

Respiration was first used in connection with the physiology of animals to designate the act of breathing. It was easy to see that a gas was inhaled and a gas exhaled. Later it was found that there was less oxygen and more carbon dioxide in the air exhaled than in that inhaled. Gradually the term respiration took on the meaning of the taking up of oxygen and the evolution of carbon dioxide by the animal body. Then it was discovered that plants also respired. The body of an animal was likened to an engine in which food was oxidized to secure energy, and the evidence of the combustion of the fuel was the evolution of carbon dioxide and water and the disappearance of gaseous oxygen. Evidently then respiration was closely tied up with the securing of energy from the food in the body cells; in fact some students came to regard respiration as the oxidative process whereby energy was made available. When the concept of respiration came to be applied to yeasts and bacteria, some difficulties were encountered. Many organisms were found that did not use free oxygen. If respiration meant the taking up of oxygen and the evolution of carbon dioxide, evidently such organisms did not respire. If, on the other hand, respiration was defined to include all processes of securing growth energy from food by oxidative processes, then the organisms did respire. Even yet physiologists are not in entire agreement as to a satisfactory definition of respiration. Most authors use the term to designate the oxidative changes produced in the cell in which oxygen serves as a hydrogen acceptor and a considerable amount of carbon dioxide is evolved. Others (and with more logic) use the term to designate any chemical process by which energy is liberated by the cell, either with or without the participation of free oxygen. In this text the term will be used in the earlier, and narrower, sense.

Fermentation is another word which has a long and rather complicated history, leading to its use by various authors with several meanings. The word comes originally from the Latin verb, *ferveo*, meaning *to boil*, from which was derived in turn *fermentum*, meaning yeast or leaven. It was first applied to alcoholic fermentation in wine making, where the evolution of gas gave the appearance of boiling. When in modern times it was discovered that alcoholic fermentation was a transformation of sugar into alcohol and carbon dioxide due to the activity of microorganisms in the absence of oxygen, fermentation was defined by Pasteur as *life without oxygen*. This definition served very well to include such changes as the lactic acid transformation of sugar, although there was no accompanying production of gas (carbon dioxide). In others words, a fermenting liquid did not need to "boil." Then the question arose as to whether changes such as acetic acid production should be called fermentation. Here oxygen was used, little or no carbon dioxide was evolved, alcohol and not sugar was attacked. This type of chemical change gradually came to be called fermentation also. But what about nitrogenous organic compounds? Should their transformations by microorganisms also be called fermentation? Finally some authors went so far as to say that all chemical changes produced by organisms should be classified as fermentations. All this is very confusing. Perhaps a definition that would be reasonably acceptable nowadays may be phrased "Fermentation is any oxidative chemical change produced by an organism in a substrate in which some of the products of breakdown are more complex than carbon dioxide." It will be noted that this definition excludes the hydrolytic changes characteristic of digestion.

The term *dissimilation* has come into use in recent years to designate any chemical breakdown of any substance by a cell. An *oxida-*

tive dissimilation is practically the equivalent of *fermentation* as just defined.

The word *putrefaction* has likewise been variously defined. It is commonly understood to indicate decomposition by microorganisms of organic substances, particularly nitrogenous materials, in the absence of air (oxygen), and with the development of unpleasant odors.

Decay is defined as decomposition of organic matter brought about in the presence of air and without the development of unpleasant odors. In practice, it is very difficult to differentiate between putrefaction and decay. In fact, the distinction is probably largely quantitative and not qualitative.

Ferments. A great advance was made in the science of microbiology when it was definitely proved by Pasteur and others that organisms such as yeasts are capable of setting up fermentations and transforming organic compounds. Before Pasteur's proposal it was believed that any organisms which might be found in fermenting liquids were wholly incidental and not the cause of the fermentation, which was held to be strictly chemical in nature and causation. The proof that living organisms do cause fermentations induced biologists to classify ferments, that is, all things capable of inciting fermentation, into two groups, *organized* and *unorganized*. The *organized ferment* was defined as a living cell which produced fermentation; the *unorganized ferment* was defined as any product of a living cell capable of bringing about fermentation. In the first group (or organized ferments) were included the yeasts producing alcoholic fermentations, the lactic acid bacteria, the acetic acid bacteria, and others, while in the second group (or unorganized ferments) were included the digestive enzymes such as the pepsin and rennin of the stomach. These unorganized ferments are now generally termed *enzymes*. Finally, proof became sufficient to lead to the conclusion that most chemical changes brought about directly or indirectly by living cells are in fact induced by enzymes. In some cases the enzymes are liberated from the cell and produce their changes in the medium surrounding the organism. In other cases the enzyme remains in the cell and is more or less intimately bound up with the protoplasm of the cell. The terms, organized and unorganized ferments, therefore, have lost their original significance, and one now speaks of changes as brought about by *extracellular* and *intracellular enzymes*.

Catalysts Produced by Cells. Cells of all plants and animals, including microorganisms, are constantly producing substances which modify the rates at which chemical reactions proceed. For example, an organism that produces an acid may thereby change the hydrogen activity (pH) of the medium and even of the cell contents. The hydrogen ions may increase the rate of many reactions. For example, cane sugar conversion to fructose and glucose is speeded up by increasing the hydrogen ion concentration. The hydrogen ions do not become a part of the product, their concentration is not changed. The hydrogen ion is an example of a *catalyst*, that is, a substance which accelerates a chemical reaction without becoming a part of the final product. It is not used up by being used.

Living cells bring about most of the changes in their chemical and physical environment through their production of a great variety of catalysts. In order to grow and increase, the cell of even the simplest organism must produce great numbers of catalysts, the number perhaps reaching into the thousands.

ENZYMES

Most of the catalysts produced by cells belong to that category of substances called *enzymes*. Essentially, an enzyme is an organic catalyst, differing from other catalysts in the complexity of its composition and in its ease of destruction. These enzymes constitute the instruments or tools which determine the courses of the multitude of life processes.

Although it is true that enzymes merely speed up reactions that would otherwise go on more slowly, it is not true that the course of events may not be profoundly influenced by the products. For example, a complex organic compound may break down by many different paths and form a large number of disintegration products. Eventually the concentration of the compound still unchanged will come into equilibrium with that of each one of these several products. Dextrose, for example, can be dissimilated in several ways. It may be transformed into alcohol and carbon dioxide or into lactic acid. If the enzyme (or group of enzymes) which catalyzes lactic acid production is present, the equilibrium between sugar and lactic acid may be quickly achieved, the other dissimilation products are scarcely in evidence. Although it is usually stated that enzymes do not initiate chemical reactions, but merely speed them up, to be exact, the spontaneous change is often so slow as to be, in practice, non-existent, and the enzyme to all intents seemingly initiates changes that would not otherwise occur.

How Enzymes Are Named. Names are given to individual enzymes and to groups of enzymes that catalyze similar chemical changes; they have been formed in various ways. In most cases and for ease of recognition names of enzymes usually end in the suffix, *-ase*.[1] In forming names of specific enzymes this ending *-ase* is most commonly added to the name of the compound which is being changed, i.e., *maltase* catalyzes the change of maltose into dextrose, *sucrase,* the change of sucrose into glucose and fructose. In some cases this type of nomenclature has been held to be inadequate because more than one enzyme may bring about changes in maltose, so in such cases a word may be coined that gives both the substrate and the reaction product, thus *maltase* becomes *malto-glucase*. A later, and probably advisable, tendency is to name enzymes with reference to the particular component of a compound attacked; *lactase,* which hydrolyzes lactose, is found to split off the β-d galactoside part of the molecule and is termed a β-d-galactosidase. In other cases enzymes take their names from the group of compounds which they change, for example, the enzymes which hydrolyze *carbohydrates* are termed *carbohydrases,* and those which hydrolyze *esters* are *esterases*. In a few cases an older non-conforming name of an enzyme has been curtailed and *-ase* added, as in the change of rennin into *rennase*. In some instances the name of the kind of chemical change involved is modified; an hydrolysis is produced by a *hydrolase* and an oxidation by an *oxidase,* an inversion (hydrolysis) of cane sugar by an *invertase*. *Catalase,* the enzyme which catalyzes the transformation of hydrogen peroxide, takes its name from *catalyst*. Occasionally (fortunately rarely), *-ase* is added to the name of a product, not the substrate, as in *peptase*. In some cases older names applied to enzymes do not end in *-ase,* as *ptyalin* (for the salivary amylase), *rennin, trypsin,* and *pepsin*.

Enzymes, as all catalysts, tend to produce an equilibrium between the substrate and the product. The reactions which they induce are reversible; the enzymes may act synthetically as well as analytically. Sometimes the ending *-ese,* as in *anhydrese,* is used to emphasize synthetic as contrasted with analytic action.

Characteristics of Enzymes. While the *exact* chemical composition of enzymes is not known, there is much information relative to their nature. Present evidence seems to indicate that in every case the enzyme is made up, at least in part, of a protein. Some enzymes, so far as analyses thus far have revealed, consist of protein only. Enzymes of a second group consist of a protein and a metallic cation, such as magnesium, zinc, or copper. Enzymes of a third group are primarily protein, but for activity require the presence of certain other definite compounds much simpler in chemical composition than the protein portion, and usually stable with respect to temperature. The additional compounds required in this third group are termed *coenzymes* or the *prosthetic*[2] groups.

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[1] One of the first enzymes to be studied was the one present in malt, which has the property of catalyzing the transformation of starch into maltose. The Greek word *diastasis,* meaning a *separation,* was taken into the French as *diastase*. Later, the word diastase was used in French literature almost synonymously with enzyme. Although in diastase the *-ase* is not a suffix, it has been converted in modern chemistry into an ending to characterize enzyme names.

Some of the enzymes have been crystallized and determinations made of their elementary composition. All contain carbon, hydrogen, oxygen, and nitrogen, some have phosphorus or sulfur.

The enzymes are generally soluble in water and in dilute salt solution. An exception is to be found in some of the fat ferments. Enzymes are partially precipitated from solution by alcohol and by concentrated solutions of ammonium sulfate, thus resembling many other proteins. They are also readily adsorbed by colloids and solid particles. For example, filtration of a solution through a porcelain filter may remove a large proportion of the enzyme originally present. Precipitation of a protein in solution will usually carry down the accompanying enzymes at the same time. Purification of enzymes is relatively difficult, but has been rendered somewhat easier through the development of methods of differential adsorption and elution.

Like microorganisms, enzymes have temperature optima, maxima, and minima for their activities and are destroyed by high temperatures. Usually action ceases at 0° C., the optimum for most types lies between 35° and 50° C., frequently at about 40° C., and with a few exceptions they are soon destroyed at temperatures above 70° C., and almost instantaneously by boiling water. When completely dried, enzymes may withstand higher temperatures.

Studies of the effect of temperatures upon activity of enzymes are complicated by the fact that, in general, an increase in temperature increases the rate at which *catalysis produced by the enzyme* occurs, but it also more rapidly increases the *rate of destruction of the enzyme* itself.

Enzymes are relatively unstable, gradually losing their ability to catalyze when in solution even under the most favorable conditions. Some lose their activity in a very few hours after isolation, others deteriorate but slowly. Light rays, particularly the violet and ultraviolet, are destructive. Enzymes are generally sensitive to the reaction of the medium. Each enzyme has an optimum pH at which it produces the maximum rate of change. Some enzymes have an optimum as low as pH 2.5–4, some as high as pH 8–10.

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[2] *Prosthetic*, from the Greek meaning, additional.

Enzymes are also sensitive to the action of many chemical compounds. Certain chemicals such as salts of the heavy metals destroy practically all enzymes. This type of action is termed *non-specific*. Other chemicals inhibit the activity of certain enzymes only; their action is termed *specific*. Among these specific inhibitors are fluorides, cyanides, and iodoacetates. Advantage is taken of this selective inhibition; a suitable inhibitor will stop the action of certain enzymes without affecting others.

Adaptive and Constitutive Enzymes. In Chapter 8 in discussion of variation among microorganisms, it was noted that by mutation individual cells may either gain or lose the ability to bring about a particular chemical change. If the mutation proved to be advantageous to the organism, the mutants would probably multiply more rapidly than the original strain and overgrow it. For example, an organism which cannot ferment or utilize lactose may produce a lactose-fermenting mutant. If grown in a lactose broth, the mutant would have a nutritional advantage. The mutation produces a permanent change in fermentative power. Even if grown for a time in a medium without lactose, the cells will still ferment this sugar when it is added. The new strain differs from the old in that it has a new enzyme or group of enzymes.

There is another quite distinct type of cell change characteristic of adaptation to a new substrate. For example, certain yeasts will ferment the sugar dextrose but not galactose. However, when galactose is present in the medium *all* the cells gradually develop the ability to ferment galactose. When a single cell is transferred to fresh medium containing galactose, it and its progeny will ferment galactose promptly. However, when grown in a medium containing no galactose, the ability to ferment this sugar is promptly lost. Apparently, a new enzyme or group of enzymes has been developed in the presence of the sugar. Enzymes which appear in a cell only after the stimulus of the presence of a new substrate are termed *adaptive enzymes*, those which are constantly present are termed *constitutive enzymes*. The development of an adaptive enzyme by a cell is not to be regarded as a mutation.

Modes of Action of Enzymes. It has been noted above that enzymes can bring about an apparently indefinite amount of change; that is, enzymes are not used up in the using and they do not form a part of the final product of their action. This does not mean, however, that enzymes never go into combination with the substance which they ferment. It is quite probable that they first combine with the compound to be transformed, bring about their characteristic change, and are later broken off and freed to unite with fresh molecules, and so continue to function indefinitely.

Reversibility of Enzyme Action. Enzymes are useful to plants and animals not only in the digestion and breaking down of complex compounds, but also in building up the protoplasm, reserve food granules, and various cell structures. Enzymes are both analytic and synthetic. It is probable that the same enzymes which under certain conditions are able to bring about digestive changes under other conditions are able to build up complex compounds. For example, a phosphorylase may break down a complex carbohydrate or it may build it up, depending in part upon relative concentrations of the reaction products. This fact is of importance as it enables one to understand why enzymes during the life of the cell may aid that cell in building up its protoplasm and reserve food materials and under other conditions may later bring about their disintegration. Enzymes which seem primarily to function synthetically during the life of the cell may after its death bring about autolysis or self-digestion.

Intracellular and Extracellular Enzymes. It has already been noted in the preceding chapter that enzymes may be divided into two classes, intracellular and extracellular. The former are present only within the cell and are never given off during its life. Even after the death of the cell it is very difficult in many cases to secure a solution of the enzyme. Extracellular enzymes, on the other hand, are excreted from the cell, and may bring about their characteristic changes outside of the cell wall.

Role of Coenzymes and Activators. Some enzymes require the presence of certain cations in the medium, some are activated by hydrogen ions, others by certain salts, as sodium chloride, others by calcium, magnesium, manganese, or other cations. In a considerable number of other enzymes there must be present some one of several relatively stable organic compounds, termed coenzymes or prosthetic groups.

The subject of the constitution and functions of the coenzymes is relatively complicated chemically, and one in which research is very active. Even the names of these compounds are not too well fixed. Here will be listed the names of those coenzymes which are best known, the type of enzymes with which they are associated, and the general nature of the reaction. In the discussion of the enzymes themselves later in this chapter there will be further references to the coenzymes.

Iron porphyrin or hematin, a complex compound related to the iron-containing component of blood hemoglobin, is a component of enzymes such as catalase and peroxidase that decompose hydrogen peroxide or oxidize organic compounds in the presence of hydrogen peroxide.

Flavin mononucleotides and dinucleotides are likewise complex compounds which function as prosthetic groups or coenzymes in many of the enzymes which catalyze oxidations.

Diphosphothiamin is the coenzyme in a group of enzymes which catalyze transformation of pyruvic acid into various substances.

Several enzymes contain various nicotinamide nucleotides, and are active as dehydrogenases.

Still other coenzymes are glutathione, adenylic acid, and pyridoxal orthophosphate.

Enzymes may be divided into three principal classes on the basis of their method of action. These classes are separated on the basis of the substrate (material acted upon by the enzyme). The primary groups are the hydrolases, the phosphorylases, and the oxidases or desmolases.

Hydrolases and Anhydreses. The hydrolases are typically digestive enzymes: they are frequently extracellular. They are hydrolytic enzymes; that is, they bring about decomposition by the incorporation of water, usually resulting in a splitting into simpler molecules. A typical example is the inversion of cane sugar by invertase.

$$\underset{\text{Saccharose}}{C_{12}H_{22}O_{11}} + H_2O \xrightarrow{\text{Invertase}} \underset{\text{Glucose}}{C_6H_{12}O_6} + \underset{\text{Fructose}}{C_6H_{12}O_6}$$

The hydrolases may under other equilibrium conditions catalyze syntheses; enzymes which cause syntheses by withdrawal of water are sometimes termed *anhydreses*. The hydrolases may be subdivided into four main groups designated as the *esterases* (or *lipases*), the *carbohydrases*, the *nucleases*, *amidases* and *deaminases*, and the *coagulases*. In general these names indicate the type of material acted upon by the several groups of enzymes. The esterases or lipases hydrolyze the esters. The carbohydrases split the carbohydrates. The amidases and proteases hydrolyze or digest proteins and allied compounds. The coagulases bring about coagulation of protein or other substances in solution. These will be discussed in order.

Esterases. These are enzymes which catalyze the hydrolysis of esters. The latter are compounds which upon hydrolysis are converted into an acid and an alcohol or carbohydrate. With organic acids the reaction may be written

$$\mathrm{R{\cdot}COOR' + H_2O \rightleftharpoons R{\cdot}COOH + R'OH}$$

in which R·COOH may represent any organic acid and R′OH may be a simple alcohol, as C_2H_5OH, a polyalcohol as glycerol ($C_3H_5(OH)_3$), an aromatic alcohol as inositol ($C_6H_{12}O_6$), or a carbohydrate. The acid may also be either sulfuric, phosphoric or compounds of these.

A *lipase* is an esterase which hydrolyzes a fat into fatty acid and glycerol, for example, stearin takes up three molecules of water and is hydrolyzed as illustrated:

$$\underset{\text{Stearin}}{(C_{17}H_{35}CO_2)_3C_3H_5} + 3H_2O \xrightarrow{\text{Lipase}} \underset{\text{Stearic acid}}{3C_{17}H_{35}COOH} + \underset{\text{Glycerol}}{C_3H_5(OH)_3}$$

The initial step in the decompositions of fats is usually of this nature. Lipases are produced by a few microorganisms.

Other esterases known to be produced by microorganisms are the *phosphatases* of many types which hydrolyze esters of phosphoric acid (including *lecithinase* which hydrolyzes lecithin), *chlorophyllase* which hydrolyzes chlorophyll to a chlorophyllide and phytol, *tannase* which changes tannin to gallic acid and glucose, *pectase* which converts pectin to pectic acid and methanol, and *sulfatase* which hydrolyzes esters of sulfuric acid.

Carbohydrases. Enzymes capable of hydrolyzing the polymers of carbohydrates are produced by many microorganisms as well as by most higher plants and animals. Carbohydrates constitute a large proportion of the food of plants and animals, and the digestion and utilization of this food is usually dependent upon the presence of such enzymes in the alimentary tract. The carbohydrases may be discussed in four groups: (1) those which hydrolyze disaccharides, (2) those which hydrolyze trisaccharides and tetrasaccharides, (3) those hydrolyzing glucosides other than disaccharides, (4) those hydrolyzing polysaccharides. The first and fourth of these groups are of much economic importance.

The enzymes which hydrolyze the disaccharides transform the sugar into two molecules of a monosaccharide.

$$\underset{\text{Disaccharide}}{C_{12}H_{22}O_{11}} + H_2O \xrightarrow{\text{Enzyme}} \underset{\text{Monosaccharide}}{C_6H_{12}O_6} + \underset{\text{Monosaccharide}}{C_6H_{12}O_6}$$

The enzyme *maltase* converts malt sugar or maltose into glucose. This enzyme is produced by certain bacteria and molds and by many species of yeasts. *Sucrase* hydrolyzes a molecule of cane sugar into one molecule of glucose and one of fructose. This enzyme is produced by a few bacteria, by many molds, and by some yeasts. The enzyme *lactase* converts lactose or milk sugar into dextrose and galactose; *cellobiase* produces two molecules of glucose from cellobiose. The enzyme which hydrolyzes a disaccharide such as sucrose is not necessarily specific for the one sugar. There are, in fact, two distinct sucrases which yield identical products but have different paths of action. Although sucrose is hydrolyzed to glucose and fructose, one may think of this sugar either as a glucoside of fructose or as a fructoside of glucose. One type of sucrase may be termed a *fructosidase* because it hydrolyzes fructosides, the other a *glucosidase* because it hydrolyzes glucosides. That there are the two sucrases was revealed when it was found that sucrase from one source would split off fructose from raffinose, while the other would not. Raffinose is a trisaccharide consisting of fructose, glucose, and galactose (a fructosylglucosylgalactoside). It is evident that one kind of sucrase is also a *raffinase;* the activity of the enzyme is determined by the kind of simple sugar it can remove. However, the simple sugars exist in these compounds in one of several forms. The enzyme is specific for one of these. A more accurate, but from the standpoint of the non-specialist, a more complicated system of naming the enzymes is being evolved. For example, the disaccharide lactose when hydrolyzed yields β-d-galactose and β-d-glucose. Lactase is a β-d-galactosidase, and will hydrolyze other β-galactoside compounds. Upon hydrolysis melibiose likewise yields galactose and glucose, but the galactose part is in the form of α-d-galactoside, and melibiase is an α-d-galactosidase which can hydrolyze melibiose and raffinose but not lactose.

Enzymes (*glucosidases*) capable of decomposing glucosides (compounds which hydrolyze into glucose and compounds other than carbohydrates) are produced by many organisms. The reaction may be illustrated by the hydrolysis of amygdalin, the essential oil of bitter almonds, by the enzyme *emulsin.*

$$\underset{\text{Amygdalin}}{C_{20}H_{27}O_{11}N} + 2H_2O \xrightarrow{\text{Emulsin}} \underset{\text{Benzaldehyde}}{C_7H_6O} + HCN + \underset{\text{Glucose}}{2C_6H_{12}O_6}$$

Emulsin is a β-glucosidase and will hydrolyze other compounds such as the disaccharides cellobiose and gentiobiose, which are β-glucosides, and is in consequence also both a *cellobiase* and a *gentiobiase.*

The polysaccharides include those carbohydrates having the general formula $(C_6H_{10}O_5)_n$. Various members of this group are hydrolyzed by different enzymes: *starch* and *glycogen* are hydrolyzed by enzymes termed *diastases* or *amylases.* The starch molecule is exceedingly complex and breaks down by a succession of hydrolytic cleavages into dextrins, and the dextrins are for the most part decomposed into maltose. There are two principal types of starch in plants: frequently they are present in mixtures. For example, cornstarch contains two different starches, amylose and amylopectin. The former is apparently a compound with long straight chains of glucose residues, the chains of variable length, perhaps from a hundred to many hundred glucose residues in length. This type of starch becomes blue in the presence of iodine. The other starch is amylopectin, apparently a much-branched chain of glucose residues which becomes red in presence of iodine. Glycogen resembles amylopectin in consisting of branched chains and giving a red-brown color with iodine. Two kinds of amylase are usually differentiated, an *α-amylase* found in malt, certain molds and bacteria, as well as in some of the digestive juices of the body. It hydrolyzes starch largely to dextrins, with little or no maltose. *β-amylase* is present in most starchy plants and hydrolyzes starch to the disaccharide maltose.

Amylases are produced by many molds, by a few bacteria, and by none of the common species of true yeasts. These enzymes (often called diastases or simply *diastase*) are also very commonly found in the higher plants, particularly in the seeds, the leaves, and the portions of the plant where starch is stored as food. The conversion of starch into maltose by the amylase derived from certain molds has proved to be of considerable importance in the preliminary treatment of starch in the manufacture of industrial alcohol. Amylases are also produced by several species of the genera *Bacillus* and *Clostridium*.

The polysaccharide cellulose is hydrolyzed by the enzyme *cellulase*. A few bacteria, and many species of molds, are known to produce cellulase. They are the forms which are active in the decay and decomposition of plant remains in nature. They are also important in the rumen and cecum of herbivorous animals. Like starch, cellulose hydrolyzes to smaller fragments and finally to the disaccharide *cellobiose*. Inulin is hydrolyzed by the enzyme *inulase* into fructose (levulose). This enzyme is known to be secreted by certain molds and a few bacteria, such as the pneumococcus. Inulin is a carbohydrate found in the roots and stems of a few plants. *Cytase* is an enzyme which has been found capable of hydrolyzing certain of the hemicelluloses. The hemicelluloses differ from the true cellulose in that their hydrolytic products, instead of being dextrose, are generally mannose, galactose, or one of the pentoses. They are, in other words, chiefly mannans, galactans, and pentosans.

The enzyme *lysozyme* is of interest in bacteriology because it is able to dissolve (hydrolyze) the cell membranes of certain bacteria. It has been found in certain animal secretions such as tears and in egg white, also in some molds and in the latex of certain plants, as in the fig. One organism, *Micrococcus lysodeikticus* is peculiarly sensitive, and its lysis (solution) has been carefully studied. The fact that little is known of the chemical composition of the cell walls of this organism makes the exact nature of the change produced by the enzyme somewhat uncertain. On the assumption that it hydrolyzes a mucopolysaccharide, it is placed with the mucolytic enzymes.

Hyaluronidase (*invasin*) is likewise a mucolytic (mucin-dissolving) enzyme which hydrolyzes hyaluronic acid. It was first given the name *spreading factor* because it seemed to facilitate the spread of disease bacteria from the site of infection through the surrounding tissues. Apparently it disintegrated the tissues and separated the cells to allow a ready growth and penetration by microorganisms. Later it was found that there are in addition to hyaluronidase other spreading factors. The connective tissue which in the body binds the cells together is made up largely of a highly polymeric substance called *hyaluronic* acid. This is also the ground substance of cartilage, bone, testicular tissue, lymphatics, and the jelly-like material of the umbilical cord. A closely related compound is found in the capsular material of many microorganisms. It is a complex polymer, mucilaginous or jelly-like in consistency. It may be hydrolyzed into its repeating units (building blocks) which no longer resemble glue in their ability to bind together or to encapsulate. The repeating unit is glucuronido-N-acetylglucosamine, which in turn may be hydrolyzed into glucuronic acid and acetyl glucosamine. The glucuronic acid is glucose with a terminal carboxyl (—COOH) group, and acetylglucosamine has attached to it the acetyl ($CH_3CO—$) and the amino (NH_2—) groups. Essentially, hyaluronic acid is a complex polysaccharide in which each of the constituent glucose residues has been modified. The hyaluronic acid (sometimes termed an acid mucopolysaccharide) is therefore related chemically to such substances as the mucins secreted by the mucous cells of the body, to the nitrogenous or mucoid capsular materials produced by many organisms and to the chitin which constitutes the material of the hard skeleton of insects and arthropods generally. Various disease-producing bacteria are known to produce hyaluronidase in the animal tissues which they invade.

The materials which bind most plant cells together in the middle lamella and which constitute outer layers of plant cell walls, particularly in fruits, are the pectins, or better, the *pectic substances*. They resemble carbohydrates, but upon hydrolysis they yield *galacturonic acid* in addition to sugar. In many cases the carboxyl radical is esterified with CH_3, or neutralized by a base such as calcium. The protopectin may be hydrolyzed by *protopectinase* and rendered soluble. This enzyme is produced by many bacteria, and fungi; it is found in some fruits. The enzyme hydrolyzes the protopectin to polygalacturonic acid esters, termed pectinic acids, colloidal in nature and capable of forming gels with sugar and acid. This enzyme, by solution of the protopectin which binds plant cells, causes them to fall apart, as in the plant diseases black rot of cabbage and carrot rot.

The pectinic acids may be further hydrolyzed by *pectinase*, an enzyme found likewise in many bacteria and fungi, into galacturonic acid or its methyl ester. Pectins may also be acted upon by the enzyme *pectase* which is an *esterase* and produces pectic acids and methyl alcohol.

Amidases are enzymes which by addition of water break the linkage between carbon and nitrogen in organic compounds. One of the best known of such enzymes is *urease* which hydrolyzes urea to ammonium carbonate.

$$\underset{\text{Urea}}{(NH_2)_2CO} + 2H_2O \xrightarrow{\text{Urease}} \underset{\text{Ammonium carbonate}}{(NH_4)_2CO_3}$$

This enzyme is produced by certain bacteria, molds, and other fungi, and is found in many higher plants, particularly legumes. *Asparaginase* hydrolyzes *l*-β-asparagin to *l*-aspartic acid

$$\underset{\text{Asparagin}}{CONH_2 \cdot CH_2 \cdot CHNH_2 \cdot COOH} + H_2O \xrightarrow{\text{Asparaginase}}$$

$$\underset{\text{Aspartic acid}}{COOH \cdot CH_2 \cdot CHNH_2 \cdot COOH} + NH_3$$

Asparagine is often used in preparation of media as a suitable nitrogen source. The enzyme is found in bacteria and yeasts as well as in higher plants.

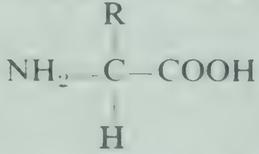
The *nuclein deaminases* catalyze the removal of an amino group with replacement by oxygen. *Adenosine deaminase* catalyzes the addition of water to adenosine with production of the sugar ribose and adenine. The adenine in turn may be hydrolyzed to ammonia and hypoxanthine by *adenase*.

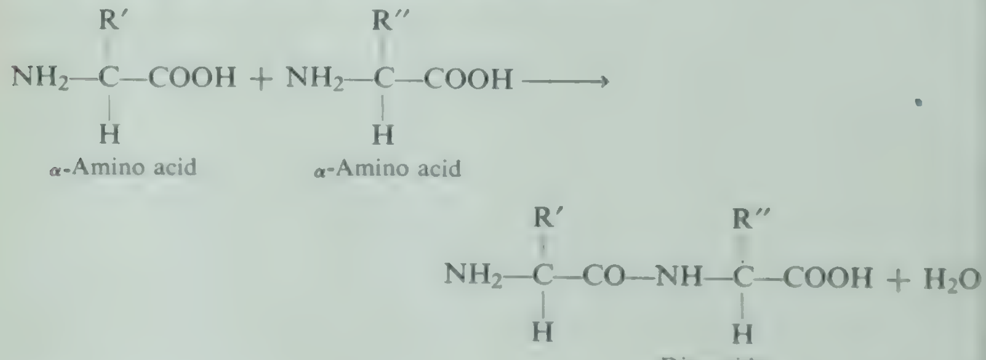
Of special significance are several enzymes (*nucleases*) which catalyze the hydrolysis of nucleic acids into mononucleotides. Two nucleic acids have been most studied. Ribonucleic acid (usually extracted from yeast) when hydrolyzed breaks down into four mononucleotides, consisting in each case of a compound which when hydrolyzed in turn yields phosphoric acid, the pentose sugar ribose and an organic base; the four bases, one for each nucleotide, are guanine, uracil, cytosine, and adenine. The enzyme which hydrolyzes ribonucleic acid is *ribonuclease*. A second important nucleic acid originally prepared from the thymus gland is thymonucleic acid, or desoxyribonucleic acid which is split into four nucleotides by *desoxyribonuclease*, each of which may be further hydrolyzed to phosphoric acid, desoxyribose and one of four bases, adenine, guanine, cytosine, and thymine. These nucleic acids and their enzymes are of special interest in studies of the internal structure of the bacterial cell. That the marked tendency of the bacterial cell to stain intensely with basic dyes may be due to the presence in the cytoplasm of ribonucleic acid. By use of ribonuclease it is possible to remove this nucleic acid quite completely, leaving the desoxyribonucleic acid characteristic of the nucleus intact. The latter can then be stained and observed microscopically to advantage.

The *desaminases* are enzymes which hydrolyze amino acids with production of hydroxy acids and ammonia. The reaction may be illustrated by the transformation of the amino acid alanine into lactic acid and ammonia.

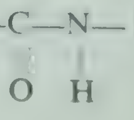
$$\underset{\text{Alanine}}{CH_3 \cdot CH(NH_2) \cdot COOH} + H_2O \xrightarrow{\text{Desaminase}} \underset{\text{Lactic acid}}{CH_3 \cdot CHOH \cdot COOH} + NH_3$$

Proteolytic Enzymes. Proteins are hydrolyzed by several enzymes through a series of breakdowns to simpler compounds. The protein molecule is exceedingly complex; it hydrolyzes through the steps of proteoses, peptones, peptides, and amino acids. The group of enzymes termed *proteinases* carry the change to peptides; the *peptidases* are primarily responsible for the hydrolysis of the peptides to amino acids, and the *desaminases* noted above free ammonia from the amino acids. The proteinases and peptidases overlap somewhat in their action.

Proteins are very complex compounds, made up of long chains of hundreds or even thousands of α-amino acid residues that are held together by peptide linkages. The α-amino acids all have the general formula  $NH_2-\overset{\displaystyle R}{\underset{\displaystyle H}{\overset{|}{\underset{|}{C}}}}-COOH$ in which R may be any one of twenty or more organic radicals. A peptide linkage results from the union of two amino acids with elimination of water.

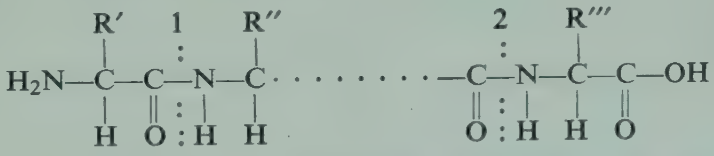


$$\underset{\alpha\text{-Amino acid}}{NH_2-\overset{R'}{\underset{H}{C}}-COOH} + \underset{\alpha\text{-Amino acid}}{NH_2-\overset{R''}{\underset{H}{C}}-COOH} \longrightarrow \underset{\text{Dipeptide}}{NH_2-\overset{R'}{\underset{H}{C}}-CO-NH-\overset{R''}{\underset{H}{C}}-COOH} + H_2O$$

The carbon-nitrogen bond in  $-\underset{O}{\underset{\|}{C}}-\underset{H}{\underset{|}{N}}-$ is the peptide linkage. The proteolytic enzyme breaks the link between the carbon and nitrogen by the addition of water.

Proteinases are sometimes called *endopeptidases* because they can attack the long chain of the protein. They are of several types, *pepsin* which acts best in a somewhat acid medium (pH 2.0 to 4.0) *trypsin* in a somewhat alkaline medium (pH 8.0 to 9.0), *papain* and other proteinases from higher plants in a medium slightly acid (pH 5.0). Enzymes resembling those of each group have been reported from microorganisms. However, some of the bacterial proteinases have an optimum of pH = 7.0–8.0, and may constitute a distinct group. Microorganisms are responsible for the spoiling of meats and other protein foods. They bring about the liquefaction of the curd in coagulated milk, the liquefaction of gelatin, the digestion of egg albumen and blood serum, and many other changes. These enzymes are also important in bringing about *autolysis* or *self-digestion*. Practically all cells contain these enzymes in small quantities and after death they hydrolyze the proteins of the protoplasm.

The enzymes first designated as *erepsins* are unable to attack the native proteins, they hydrolyze peptones and peptides, with the ultimate formation of amino acids. There are many enzymes belonging to this group; they are now generally termed *peptidases*. Among those which have been described, the following are best known. A typical polypeptide consists as noted above of a chain of amino acid residues, with a free carboxyl at one end and not at the other. Such a polypeptide may be represented as follows:



$$\begin{array}{cccccccccccccc}
 & R' & & & 1 & & R'' & & & 2 & & R''' & & \\
 & | & & & \vdots & & | & & & \vdots & & | & & \\
H_2N- & C & - & C & \vdots & -N- & C & \cdots\cdots & -C & \vdots & -N- & C & -C & -OH \\
 & | & & \| & \vdots & | & | & & \| & \vdots & | & | & \| & \\
 & H & & O & : & H & H & & O & : & H & H & O &
\end{array}$$

An *amino polypeptidase* adds water at the bond numbered 1, attacking the amino acid end with a free amine group but no carboxyl. The hydrolysis results in an amino acid and the development of an amino group on the adjoining amino acid residue, which in turn may be removed. A *carboxypolypeptidase* is one that begins at the other end of the chain, with the amino acid residue having a free carboxyl and splits the molecule at bond number 2, with production of a molecule of amino acid and production of a new carboxyl at the end of the polypeptide. The latter is then ready for the removal of another amino acid. A *dipeptidase* is one which hydrolyzes a dipeptide to two molecules of amino acid. Still other types of peptidases have been described which hydrolyze peptides with certain end amino acid residues, as *prolinase,* freeing proline.

Coagulases. These are the enzymes which transform soluble native proteins into such form that they are readily precipitated or coagulated. The best known of these is *rennin* (*rennet*) which transforms the casein (or caseinogen) of milk into paracasein (casein) and coagulates the milk. This type of coagulation, although favored by some acidity, is to be sharply distinguished from the curdling of milk by acid. Rennet is usually prepared from the fourth stomach of the calf and is used for cheese making. Many plants, as the fig and milkweed, also produce milk coagulases, as do many bacteria and molds. An enzyme, produced by certain spore-producing bacteria and by certain molds which coagulates egg yolk, has been named *vitellase.* Bacterial enzymes (*coagulases*) produced by some bacteria are known to clot blood plasma.

The Phosphorylases. *Phosphorolysis* is that process of breaking down certain organic compounds through the catalytic action of enzymes termed *phosphorylases.* These enzymes have been found to be very common and very important, particularly because they bring about equilibria which induce syntheses. Phosphorylases are therefore significant not only in breaking down complex polymers and other compounds, but in building them up as well. The breakdown and synthesis of sucrose may serve as an example. It will be recalled that sucrose may be hydrolyzed into glucose and fructose by the enzyme sucrase in the presence of water.

$$\underset{\text{Sucrose}}{C_6H_{11}O_5{-}O{-}C_6H_{11}O_5} + H_2O \overset{\text{Sucrase}}{\rightleftarrows} \underset{\text{Glucose}}{C_6H_{12}O_6} + \underset{\text{Fructose}}{C_6H_{12}O_6}$$

The action of a *phosphorylase* is somewhat analogous, it catalyzes the addition of a molecule of phosphoric acid with the formation of glucose-1-phosphate and fructose.

$$\underset{\text{Sucrose}}{C_6H_{11}O_5{-}O{-}C_6H_{11}O_5} + H_3PO_4 \overset{\text{Phosphorylase}}{\rightleftarrows} \underset{\text{Glucose-1-phosphate}}{C_6H_{11}O_5{-}O{-}PO_3H_2} + \underset{\text{Fructose}}{C_6H_{12}O_6}$$

Not only sucrose but also polysaccharides such as starch and glycogen may be split by this same mechanism, and by a reverse process starch or glycogen may be synthesized with accompanying dephosphorylation, that is, by removal of phosphoric acid.

The phosphorylases are found to be involved in a great number of enzymatic changes; increase in knowledge of the mechanism of their operation has opened up a whole new field of opportunity for study of the metabolic changes which go on in living cells. Among the results that have proved most helpful in understanding these changes is the fact that the formation of certain phosphates binds a large amount of energy which must be derived from some accompanying chemical change. Such phosphate compounds have this energy stored in the phosphate bond, which is said to be "energy-rich." They are reactive, and phosphorylase catalyzes the transfer of the phosphate radical to other compounds. As an example of the probable sequence of events in one series of transformations, we may follow the steps in the synthesis of sucrose. First, glucose in the presence of the specific phosphorylase *hexokinase* is phosphorylated by transfer of a phosphate radical from adenosine triphosphate.

$$\underset{\text{Glucose}}{C_6H_{12}O_6} + \text{adenosine triphosphate} \xrightarrow{\text{Hexokinase}}$$

$$\underset{\text{Glucose-6-phosphate}}{C_6H_{11}O_4\text{—}O\cdot PO_3H_2} + \text{adenosine diphosphate}$$

The phosphoric acid radical is then transferred from position 6 in the sugar to position 1, through a change catalyzed by another phosphorylase, *phosphoglucomutase,* and glucose-6-phosphate becomes glucose-1-phosphate. This in turn in the presence of still another phosphorylase and fructose is dephosphorylated and unites with the fructose to form sucrose and phosphoric acid.

Oxidases and Reductases. Probably all enzymatic changes involve some degree of oxidation or reduction of the substrates on which they work. The hydrolyses of compounds usually involve little energy change. Phosphorolysis may involve considerable energy change and the phosphorylases are likewise oxidizing and reducing in their activity. There is another group of enzymes termed oxidases in which the oxidations and reductions are neither hydrolytic or phosphorolytic. They may collectively be termed the *oxidases.* However, whenever there is an oxidation there must be a simultaneous reduction, so that these same enzymes are also *reductases.* These enzymes are of special importance to the cell in that they are basic in respiration and in many fermentative changes. Inasmuch as they are significant in the breakdown of organic compounds they have sometimes been termed the desmolytic enzymes or *desmolases.* The hydrolytic changes produced by the hydrolases discussed above release or bind relatively small amounts of energy; they are primarily digestive in function. The oxidases, on the other hand, frequently release by their action considerable quantities of energy. The hydrolytic enzymes are frequently extracellular, whereas the oxidative enzymes are usually intracellular, the cell making use of the energy released by their activity in synthesis, heat production, motion, light production, etc.

Reactions in which one substance is oxidized and the other is reduced are termed *coupled reactions*. For example, some bacteria are able to couple the reduction of sulfate to sulfide with the oxidation of organic compounds.

For the most part the active oxidases of microorganisms oxidize organic compounds, more rarely inorganic. It should be clearly held in mind that oxidation of an organic compound may be brought about in at least two distinct ways; either by the addition of oxygen to the compound or by the removal of hydrogen. For example, ethane may be oxidized to ethyl alcohol by the addition of oxygen:

$$\underset{\text{Ethane}}{C_2H_6} + O \rightarrow \underset{\text{Ethyl alcohol}}{C_2H_5OH}$$

It may also be oxidized to ethylene by the removal of hydrogen:

$$\underset{\text{Ethane}}{C_2H_6} \rightarrow \underset{\text{Ethylene}}{C_2H_4} + H_2$$

Any enzyme which catalyzes the addition of oxygen or the removal of hydrogen is to be regarded as an oxidase, and any which removes oxygen or adds hydrogen is a reductase. It is apparent further that if hydrogen is added to one compound it must be removed from another.

The classification of the oxidases and reductases may be made upon several different bases. That adopted by Tauber (1949) will be used. The enzymes are grouped under the headings of *oxidases* which produce oxidations by transfer of oxygen, and the *dehydrogenases* which transfer hydrogen. There are also some oxidizing enzymes which do not classify readily under either heading.

The true *oxidases* contain either the complex iron-porphyrin related to the iron-containing hematin derivable from the red coloring matter of blood, or they contain copper in the active or prosthetic group. Several should be noted. The enzyme *catalase* is produced by most, though not by all, microorganisms that grow in the presence of air. It is usually absent in the anaerobic bacteria. It catalyzes the decomposition of hydrogen peroxide into water and oxygen.

$$\underset{\substack{\text{hydrogen}\\ \text{peroxide}}}{2H_2O_2} \xrightarrow{\text{Catalase}} 2H_2O + O_2$$

It has been found that hydrogen peroxide is formed by many cells; it is also poisonous to them. Apparently the presence of the catalase causes the removal of toxic material as rapidly as it is formed. The catalase is usually regarded as a reductase, its primary activity is that of removal of oxygen. Some have suggested that aerobic bacteria are those which are able to grow in the presence of air (oxygen) because by production of catalase they are able to destroy the hydrogen peroxide that is formed, while anaerobes are such because when brought in contact with oxygen they produce the poisonous hydrogen peroxide and, being without catalase, cannot destroy it. While this seems to be true for most microorganisms, there are some species which can grow in the presence of air without formation of catalase, in some cases probably because the amount of peroxide produced is small and the cells are somewhat resistant, in other cases neither peroxide nor catalase is formed.

Catalase is one of the most active enzymes known. It has been calculated that one molecule of catalase will catalyze the decomposition of 2,600,000 molecules of H_2O_2 per minute at 0° C. Catalase is produced by many cells in quantities far greater than the amount needed for protection against hydrogen peroxide formed in the cell metabolism. Recent work seems to indicate that catalase in the cell in the presence of other oxidases which oxidize water to hydrogen peroxide catalyzes the oxidation of various compounds, as for example, ethyl alcohol to acetic acid.

Peroxidase resembles catalase in containing hematin. This enzyme catalyzes the reduction of hydrogen peroxide and the coupled oxidation of other organic compounds. The reaction may be illustrated:

$$\underset{\substack{\text{Reduced}\\ \text{compound}}}{RH_2} + H_2O_2 \xrightarrow{\text{Peroxidase}} \underset{\substack{\text{Oxidized}\\ \text{compound}}}{R} + 2H_2O$$

In some cases the substrate becomes colored when oxidized. For example, pyrogallol is oxidized to purpurogallin, a purple compound.

The intensity of color produced may be used as an index to the concentration of the peroxidase. This enzyme is produced by some bacteria; it is abundant in parts of certain higher plants.

Cytochromes. The importance of the cytochromes first discovered by MacMunn (1886) was emphasized by Keilin (1939). They were named cytochromes (from the Greek for cell color) because they were pigments and were to be found generally in living cells. Of the three cytochromes a, b, and c, the cytochrome c has been most studied. Like the catalase and peroxidase it contains hematin (iron-porphyrin). It is readily reduced by various agents such as the hydrosulfites and ascorbic acid and by an enzyme called *cytochrome reductase*, whereupon the iron of the compound is found to be in the reduced or ferrous state. The reduced cytochrome may be oxidized by one of several oxidases, particularly by *cytochrome oxidase*, when the iron is oxidized to the ferric form. The reduced cytochrome oxidase is thus oxidized in the presence of molecular oxygen. The several cytochromes and their associated enzymes apparently play an important role in the dehydrogenation of nutrients in cell metabolism and in the use of oxygen as a hydrogen acceptor. The combination of cytochromes with oxidases and reductases, termed the cytochrome system, are responsible for a large part, perhaps two thirds of the oxygen consumption of cells. The overall action of cytochromes may be illustrated as follows.

> Reduced substrate (RH_2) + 2 cytochromes (Fe^{+++}) $\longleftrightarrow$ Oxidized substrate (R) + $2H^{+}$ + 2 reduced cytochromes (Fe^{++}).
> The cytochrome functions as an electron acceptor in the oxidation of the substrate. The cytochrome oxidase then catalyzes the oxidation of the reduced cytochrome, with oxygen acting as a hydrogen acceptor.
> 2 reduced cytochromes (Fe^{++}) + $\frac{1}{2}O_2$ + $2H^{++}$ $\longleftrightarrow$ 2 cytochromes (Fe^{+++}) + H_2O

Most bacteria which will grow in contact with oxygen contain one or more cytochromes, though not all have been shown to contain the cytochrome oxidase. Strict anaerobes apparently contain no cytochrome, and no cytochrome oxidase.

Enzymes Containing Copper. The oxidases just discussed were iron-containing through content of hematin related to the hemoglobin of blood. In certain animals, as certain mollusks and arthropods, the blood pigment contains copper rather than iron, and the copper-containing hemoglobin functions as a respiratory pigment. It is now known that somewhat similar compounds constitute the prosthetic groups of certain oxidizing enzymes.

Ascorbic acid oxidase is found in certain plants. It oxidizes the reduced form of ascorbic acid (vitamin C) to the oxidized form, requiring free oxygen.

$$\underset{\substack{\text{Reduced}\\\text{ascorbic acid}}}{C_6H_8O_6} + H_2O + \tfrac{1}{2}O_2 \xrightleftharpoons{\substack{\text{Ascorbic acid}\\\text{oxidase}}} \underset{\substack{\text{Dehydroascorbic}\\\text{acid}}}{C_6H_{10}O_8} \rightleftharpoons C_6H_6O_6 + 2H_2O$$

The hydrogen unites with oxygen. The enzyme is present in many plant tissues.

Tyrosinase is an oxidase produced by many plant cells, including many fungi and molds and some bacteria. It catalyzes the oxidation of the amino acid tyrosine through the initial stages of the transformation into the black or dark polymer melanin. The darkening of the cut surface of fruits and plants, particularly the darkening of the cut surfaces of certain mushrooms and the browning of culture media in which certain microorganisms are grown in the presence of tyrosine, are due to the enzyme tyrosinase. Related enzymes, sometimes termed *phenolases,* catalyze the oxidation of related compounds.

Dehydrogenases. These oxidizing enzymes catalyze the removal of hydrogen. They apparently activate hydrogen of the substrate so it may be transferred to another compound which is thereby reduced. Very rapid progress is being made in study of this field of enzyme characterization, and those enzymes noted here are but a few in a list which is constantly growing. They are of several distinct groups.

The first group of dehydrogenases requires the presence of a coenzyme which acts as the intermediate carrier of the hydrogen transferred to it by the dehydrogenase itself. Two coenzymes have been described and studied. One of these is the cozymase discovered by Harden and Young (1905), and later shown by Euler and Schlenk (1937) to be a diphosphopyridine nucleotide (also termed codehydrogenase I). Chemical study has shown it to be made up of a chain of residues as follows:

$$\text{Nicotinamide} \cdot \text{pentose} \cdot (\text{phosphoric acid})_2 \cdot \text{pentose} \cdot \text{adenine}$$

The second of the coenzymes (called codehydrogenase II) differs from the above primarily in the presence of a third phosphoric acid residue; it is a triphosphopyridine nucleotide. It is the nicotinamide residue which is reduced by the dehydrogenase. The coenzyme may then be again oxidized by transferring its hydrogen load to another component of the substrate and be ready then to be reduced by the dehydrogenase. Among the dehydrogenases that seem of special significance in microbiology are the following.

Alcohol dehydrogenase, which is found in yeasts and molds and some bacteria, catalyzes the reversible change of ethyl alcohol to acetaldehyde:

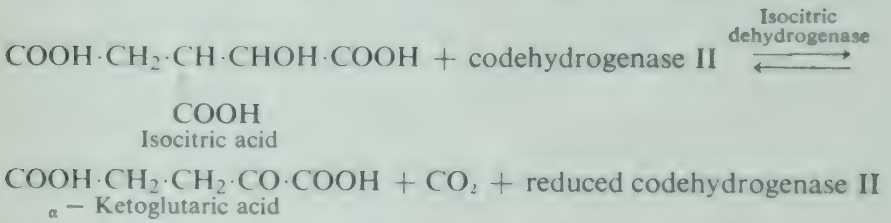
$$\underset{\text{Ethanol}}{CH_3{\cdot}CH_2OH} + \text{codehydrogenase I} \xrightleftharpoons{\text{Alcohol dehydrogenase}} CH_3{\cdot}CHO + \text{reduced codehydrogenase I}$$

Lactic Dehydrogenase. Some bacteria contain this enzyme which catalyzes the dehydrogenation (oxidation) of lactic acid to pyruvic acid.

$$\underset{\text{Lactic acid}}{CH_3{\cdot}CHOH{\cdot}COOH} + \text{codehydrogenase I} \xrightleftharpoons{\text{Lactic dehydrogenase}} \underset{\text{Pyruvic acid}}{CH_3{\cdot}CO{\cdot}COOH} + \text{reduced codehydrogenase I}$$

The lactic dehydrogenase of yeast does not require the presence of the cozymase.

One of the enzymes which requires the presence of the second codehydrogenase is worthy of mention because it illustrates one method by means of which carbon dioxide may be incorporated into an organic compound. This general problem of CO_2 assimilation will be discussed in more detail later. This enzyme is isocitric dehydrogenase which (apparently in two steps) catalyzes the production from isocitric acid of α-ketoglutaric acid and carbon dioxide.



$$\underset{\text{Isocitric acid}}{COOH{\cdot}CH_2{\cdot}\underset{\displaystyle COOH}{CH}{\cdot}CHOH{\cdot}COOH} + \text{codehydrogenase II} \xrightleftharpoons{\text{Isocitric dehydrogenase}} \underset{\alpha-\text{Ketoglutaric acid}}{COOH{\cdot}CH_2{\cdot}CH_2{\cdot}CO{\cdot}COOH} + CO_2 + \text{reduced codehydrogenase II}$$

It will be noted that the reaction is indicated as reversible, which means that under suitable conditions of concentration, carbon dioxide may be used in synthesis of a more complex organic compound.

Another group of dehydrogenases does not utilize either of the codehydrogenases but transfers hydrogen through cytochromes or other carriers to other compounds. Yeasts and bacteria produce *lactic dehydrogenases* which transform lactic acid into pyruvic acid. Certain bacteria produce a *formic dehydrogenase* which decomposes formic acid into carbon dioxide and a reduced hydrogen acceptor. In the presence of an unknown iron-containing substrate, the hydrogen appears as molecular hydrogen.

Another series of dehydrogenases contains the so-called *flavin* group, which acts as a hydrogen acceptor, as well as a protein group. Some of these can transfer the hydrogen directly to molecular oxygen, in others the cytochrome must be used as a hydrogen acceptor and carrier. These *flavoprotein* or *yellow enzymes* are so named because the oxidized flavins are yellow. The essential constituent of the prosthetic group is riboflavin (vitamin B_2) phosphate. The riboflavin acts as a reversible oxidation-reduction system. Several enzymes of this group have been found in microorganisms. One of these has been named the *"old yellow enzyme"* because it was the first of the group to be studied; it was isolated from yeast. It is a combination of riboflavin monophosphoric acid and a specific protein. This enzyme oxidizes reduced codehydrogenase and takes up hydrogen which it then transfers to molecular oxygen with formation of hydrogen peroxide.

Another is *xanthine dehydrogenase* which oxidizes xanthine to uric acid and aldehydes to corresponding acids. This enzyme catalyzes the reduction of the dye methylene blue to its colorless form in the presence of aldehydes. Bacterial *l-amino acid oxidase* oxidizes the naturally occurring *l*-amino acids to the corresponding keto acids. Certain molds, particularly members of the genera *Aspergillus* and *Penicillium* produce a *glucose oxidase* (notatin) which catalyzes the transformation of glucose to gluconic acid and hydrogen peroxide.

$$\underset{\text{Glucose}}{C_5H_{11}O_5CHO} + O_2 + H_2O \rightarrow \underset{\text{Gluconic acid}}{C_5H_{11}O_5COOH} + H_2O_2$$

This enzyme has enough antibacterial activity to lead to its inclusion among the antibiotics. Apparently, the production of hydrogen peroxide is significant in inhibiting growth of *Micrococcus aureus* even in dilutions of one to a billion.

Several additional oxidizing enzymes are significant in bacteriology but are not readily classified. One of these is the enzyme which catalyzes the chemical changes resulting in light production by microorganisms such as certain fungi and bacteria. However, the enzymes and substrates concerned have not as yet been isolated from these organisms, and most of the critical work has been with luminescent animals, particularly with a crustacean (copepod) which has a luminescent gland near the head. It has been shown that an enzyme *luciferase* oxidizes a substrate luciferin and light is emitted in the process. Certain bacteria known to oxidize uric acid may have the enzyme *uricase* which catalyzes the reaction:

$$\text{Uric acid} + O_2 + H_2O \xrightarrow{\text{Uricase}} \text{Allantoin} + CO_2 + H_2O_2$$

A group of enzymes of major significance are those which bring about decarboxylations of α-keto acids, such as pyruvic acid, in general accord with the reaction:

$$\underset{\text{Pyruvic acid}}{CH_3{\cdot}CO{\cdot}COOH} \xrightarrow{\text{Carboxylase}} \underset{\text{Acetaldehyde}}{CH_3{\cdot}CHO} + CO_2$$

This enzyme is found in yeasts and other microorganisms. Later it was found that the carboxylase could be separated into two components, neither active alone, a protein and a heat resistant fraction. When the two fractions were reunited the enzyme activity was restored. To the stable portion the name cocarboxylase was given. This was later found to be diphosphothiamin.

Certain bacteria produce amino acid decarboxylases which remove CO_2 from the carboxyl of α-amino acids with the resultant formation of the corresponding amine.

$$\underset{\text{Amino acid}}{R{\cdot}CHNH_2{\cdot}COOH} \xrightarrow[]{\substack{\text{Amino acid}\\ \text{decarboxylase}}} \underset{\text{Amine}}{R{\cdot}CHNH_2} + CO_2$$

These products may be important, as some of the amines are poisonous.

A peculiarly important enzyme is *oxalacetic carboxylase* (β-carboxylase) which catalyzes the reaction:

$$\underset{\text{Oxalacetic acid}}{COOH{\cdot}CH_2{\cdot}CO{\cdot}COOH} \xrightleftharpoons{\beta\text{-Carboxylase}} \underset{\text{Pyruvic acid}}{CH_3{\cdot}CO{\cdot}COOH} + CO_2$$

This reaction was shown to be reversible by the classic work of Wood and Werkman which constituted the first clear demonstration that carbon dioxide could be assimilated in the course of normal metabolism of cells which were neither chemosynthetic nor photosynthetic.

Several enzymes are known which add water to an organic compound without splitting the molecule. These are called either *hydrases* or *mutases*. One of these found in yeast and bacteria, *glyoxalase*, catalyzes the reaction

$$\underset{\text{Glyoxal}}{CH_3\cdot CO\cdot CHO} + H_2O \overset{\text{Glyoxalase}}{\rightleftarrows} \underset{\text{Lactic acid}}{CH_3\cdot CHOH\cdot COOH}$$

This enzyme has as a coenzyme *glutathione*.

Aldehyde mutase converts aliphatic aldehydes such as acetaldehyde by simultaneous oxidation and reduction (Cannizzaro reaction) into an equimolar mixture of acid and alcohol.

$$\underset{\text{Acetaldehyde}}{2CH_3\cdot CHO} + H_2O \xrightarrow{\substack{\text{Aldehyde}\\ \text{mutase}}} \underset{\text{Acetic acid}}{CH_3COOH} + \underset{\text{Ethanol}}{CH_3CH_2OH}$$

Hydrogenase is an enzyme which catalyzes the reduction of various substances by molecular hydrogen. For example, certain bacteria in the presence of hydrogen decolorize methylene blue. Other bacteria cannot produce this change. It has been postulated that hydrogenases might be of major significance in explaining such phenomena as formation of methane from CO_2 and H_2 and the fixation of atmospheric nitrogen. The significance of hydrogenase is still not clear.

A reading of this chapter on enzymes, even though relatively only a very few enzymes are discussed, reveals that enzymology is a highly complex branch of the general field of biochemistry. The enzymes are the essential tools used by every cell in producing the constant flow of chemical changes necessary for metabolism and the maintenance of life. For the bacteriologist, some knowledge of some of the principal groups and of some of their reactions is important, but a command of the subject is the lifetime work of the specialist.

CHAPTER 22

Chemical Changes of Economic Significance Produced by Microorganisms

The number of chemical changes that can be produced by living cells is legion. Every functioning cell is building and tearing down many compounds—all as a part of cell metabolism and cell life and activity. Even though the cells of microorganisms may seem to have a comparatively simple morphology, they are fully as complex, perhaps even more complex physiologically than many of the cells of higher plants and animals, for the range of chemical changes which must be brought about by a cell which manufactures all its food from simple compounds must be greater than those produced by a cell to which much of the food comes partly synthesized.

Only a few of the changes which are known to be produced by microorganisms can be discussed here. Some are important because of the products formed, others because there is special interest in the mechanisms involved, or because of the significant utilization of microorganisms in biochemical analyses.

ORIGIN OF THE PRODUCTS OF FERMENTATION

There are five distinct ways in which microorganisms bring about chemical changes.

Analytic Action of Extracellular Enzymes. Many organisms excrete enzymes which can bring about analytic changes in substances outside of the cell. The changes produced may be of many types. Some extracellular enzymes break down insoluble or colloidal compounds, as by digesting starch (changing it to sugars) or by digesting proteins (hydrolyzing them into proteoses and peptones). These enzymes are doubtless useful to the organism because they make food materials soluble and diffusible. However, certain substances in solution can enter while others are held back. It is not only necessary that a food substance be dissolved, but it must also be of such a nature that it can diffuse through the cell wall and ectoplast of the organism before it can be utilized as food. For example, cane sugar ($C_{12}H_{22}O_{11}$) will not pass through some plant membranes, whereas dextrose and levulose ($C_6H_{12}O_6$) are readily diffusible. Organisms living in solutions containing cane sugar, therefore, may "invert" (hydrolyze) the sugar into a mixture of dextrose and levulose which can pass into the cell.

Analytic Action of Intracellular Enzymes. Any living cell, whether plant or animal, possesses intracellular enzymes, which are essential to its life processes. Some of the products of the analytic action of these intracellular enzymes are therefore constantly being given off by active cells. The amount of these products depends very largely upon the mode of life adopted by the organisms. Those, for example, that live under anaerobic conditions and are dependent for their energy supply upon the destruction of large quantities of organic matter excrete the waste products in large quantities. Many of the economic fermentations of importance are of this character, e.g., the conversion of sugar into alcohol and carbon dioxide or into lactic or butyric acids. Some intracellular enzymes, however, are very active under aerobic conditions. Such, for example, are those of mother-of-vinegar, which convert alcohol into acetic acid.

Analytic Action of Autolytic Enzymes. All cells contain enzymes which are capable of bringing about a more or less complete digestion of the cell protoplasm upon the death of the cell. When large numbers of organisms have grown to maturity in a nutrient solution, a considerable proportion of these are destroyed and their bodies undergo partial digestion or *autolysis*. In consequence the constituents of the body of the organism go into solution. This autolysis may be of importance as in the preparation of certain vaccines.

Synthetic Action of Organisms. In some cases the intracellular and extracellular enzymes may bring about syntheses, and these synthetic products may be of economic importance. The gums and slimes, capsules, cell walls, the living material of the cell itself, and pigments, are all the result of syntheses. A detailed discussion will be found later in this chapter.

Secondary Action. The four sources outlined above constitute the primary sources of the products of action of microorganisms. In addition, the substances developed and excreted by cells may bring about secondary reactions in the environment. The production of acids may hasten the solution and the making available to crop plants of relatively insoluble soil compounds, such as those of phosphorus.

Before attempting a classification of the numerous chemical changes of economic interest originating in the several ways just described, it is well to examine with some care the changes that are brought about by microorganisms in the three simple substances, carbon dioxide, oxygen, and nitrogen. Under the heading of "Cycles in Nature" some of the changes which they undergo have already been discussed.

FIXATION OF CARBON DIOXIDE

The gas carbon dioxide is continually poured into the atmosphere as a result of combustion, respiration, and the decay of organic matter. Most animals and plants excrete this gas as one of the products of cellular metabolism. With approximately equal rapidity the carbon dioxide is removed from the air, in large part, though not altogether, as a result of fixation by living cells and is built by them into organic compounds which are used by the cells.

Photosynthetic Fixation of Carbon Dioxide. A series of brilliant discoveries has made possible explanations of some of the mechanisms which are used by cells in fixing carbon dioxide. The first of these discoveries was the finding that green plants (plants containing chlorophyll) when *properly illuminated* assimilate carbon dioxide, give off gaseous oxygen, and produce complex organic compounds, usually starch, but sometimes fats or oils. Careful study showed that the number of molecules of oxygen liberated was equal to the number of molecules of carbon dioxide fixed. Inasmuch as carbohydrates were found to be the common end product and the general formula for the simple carbohydrates may be written $(CH_2O)_n$, the overall reaction involved was conceived to be

$$CO_2 + H_2O \xrightarrow[\text{light}]{\text{Chlorophyll}} \underset{\text{Formaldehyde}}{(CH_2O)} + O_2$$

with the formaldehyde polymerizing promptly,

$$n(CH_2O) \rightarrow \underset{\text{Sugar}}{(CH_2O)_n}$$

It was concluded that the absorption of the energy of light by the chlorophyll made possible the reduction of the CO_2 to formaldehyde, but it was soon discovered that the problem is not so simple, is very complicated, so complicated in fact that proof of the exact nature of the mechanism is even now not completely at hand. For example, the above equation would seem to indicate that the oxygen freed might have come from either the CO_2 or the H_2O. Van Niel, by use of water containing an isotope of oxygen showed that all the oxygen freed came from the water.

Much of recent advance in our understanding of carbon dioxide fixation has been due to the facility with which certain unicellular organisms with chlorophyll, such as the alga *Chlorella* can be cultivated and studied in the laboratory. The process of fixing CO_2 through light and chlorophyll is called *photosynthesis*. This *photosynthetic process* is by far the most important of the methods of fixing CO_2, from the standpoint of the total amount fixed in nature. All organisms that can utilize CO_2 as the sole source of carbon may be termed *autotrophic* (self-feeding) or *prototrophic* (feeding simply).

Winogradsky (1888) called attention to the fact that there are certain pigmented bacteria which grow in the light and in the presence of hydrogen sulfide and are able to oxidize the H_2S to S_2. The work of Van Niel and associates has shown that CO_2 is fixed. Evidently there is a complex coupled reaction whereby the CO_2 is reduced and the H_2S oxidized to free sulfur. Assuming that the carbon products of the CO_2 fixation are the same as noted above for photosynthesis, the overall reaction may be written

$$CO_2 + 2H_2S \rightarrow CH_2O + S_2 + H_2O$$

In some cases the sulfur may be further oxidized to sulfates. These investigators later discovered that certain reduced organic compounds may replace hydrogen sulfide. If such a compound is represented as H_2A, then the overall reaction may be written as

$$CO_2 + 2H_2A \rightarrow CH_2O + 2A + H_2O$$

Certain green bacteria (*Chlorobacteriaceae*) and some sulfur bacteria are photosynthetic and have sometimes been considered to be forms intermediate between the true bacteria and the algae. Again it may be emphasized that the energy required for the reduction of the CO_2 is derived from light. However, some of the purple photosynthetic bacteria if supplied with suitable organic nutrients will grow and develop much as do other bacteria and without dependence upon light.

Autotrophic Non-Photosynthetic Fixation of CO_2. A second group of autotrophic organisms is *chemosynthetic* and not photosynthetic.

These bacteria do not utilize light as a source of growth energy. They reduce and fix carbon dioxide through coupled reactions in which various elements and inorganic compounds are oxidized and thereby furnish the needed energy. Winogradsky (1890) was the first definitely to identify and characterize certain bacteria as able to grow in the absence of organic compounds, to fix CO_2 and to oxidize ammonia to nitrites (*Nitrosomonas*) or nitrites to nitrates (*Nitrobacter*). These bacteria are probably more important in agriculture through their power of oxidizing ammonia and nitrites than for their ability to fix CO_2.

Winogradsky also did the first work on an important group of chemosynthetic bacteria which accompany CO_2 fixation by the oxidation of sulfur and reduced sulfur compounds. He observed that filamentous bacterium *Beggiatoa* would grow in the absence of organic matter and, in the presence of hydrogen sulfide, was able to fix carbon dioxide and to oxidize the sulfur to free sulfur, which was deposited as granules in the cell of the organism. Later, several species of bacteria belonging to the genus *Thiobacillus* were found to be able to oxidize free sulfur or compounds of sulfur and to fix carbon dioxide. One of them, *Thiobacillus thiooxidans*, will grow in a solution to which free sulfur has been added and oxidize this to sulfuric acid, producing a tenth normal solution. Evidently the energy needed for the fixation of the CO_2 must come from the oxidative change. The sulfur bacteria may be divided into those which are obligate autotrophs and those which are facultative. The latter can grow in the presence of organic compounds and do not require the presence of sulfur.

Certain bacteria, usually facultative autotrophs, are able to fix and utilize carbon dioxide in the presence of elementary hydrogen. There have also been described members of the group of iron bacteria which apparently are able to oxidize ferrous iron compounds to ferric and to use the energy in the fixation of CO_2. It should be noted that the mechanism of fixation of CO_2 is not well understood in any of the chemosynthetic forms.

Heterotrophic Fixation of CO_2. Until 1935 it was generally considered that all carbon dioxide fixation and utilization by living cells was accomplished through the growth and activity of the photosynthetic and chemosynthetic plants and microorganisms. Other forms were supposed to be quite unable to bring about this change. However, Theobald Smith (1924) had shown that *Brucella abortus* requires CO_2 for growth; other observers noted that CO_2 was either necessary or acted as an accelerant to growth in the cultivation of many bacteria. But the mechanism of its action was quite unknown. Wood and Werkman (1936) in a study of the metabolism of the propionic bacteria found that, in a closed system, which contained carbonate in the beginning, the amount of carbonate decreased. In a series of fundamental studies Werkman and his associates showed that propionic acid bacteria are able to assimilate carbon dioxide and use it in building organic compounds. They went farther and established for the first time the mechanism by which the CO_2 is fixed. They found that the number of mols of CO_2 fixed was the same as the number of mols of succinic acid produced in the process of fermentation of glycerol. Later they were able by means of the use of an isotope of carbon to determine the mechanism as

$$CO_2 + \underset{\text{Pyruvic acid}}{HCH_2{\cdot}CO{\cdot}COOH} \rightarrow \underset{\text{Oxalacetic acid}}{HOOC{\cdot}CH_2{\cdot}CO{\cdot}COOH}$$

In the presence of suitable hydrogen donators the oxalacetic acid is reduced to succinic acid

$$\underset{\text{Oxalacetic acid}}{HOOC{\cdot}CH_2{\cdot}CO{\cdot}COOH} + 4H \rightarrow \underset{\text{Succinic acid}}{HOOC{\cdot}CH_2{\cdot}CH_2{\cdot}COOH} + H_2O$$

The discovery that heterotrophic bacteria are able to fix CO_2 opened up a whole new field of investigation, and it was soon found that many cells, both of animals and plants, not only may utilize CO_2 but in fact require CO_2 for normal growth. The mechanism of fixation varies with different types of cells, but the Wood-Werkman reaction is typical of those fixations in which the carbon of the CO_2 is united with another carbon.

Another type of reaction in which CO_2 becomes an "organic" compound is through such changes as reductions of CO_2 by *Escherichia coli* to formic acid as shown by Wood (1936)

$$CO_2 + 2H \rightarrow \underset{\text{Formic acid}}{HCOOH}$$

or the production of methane from CO_2 as determined by Barker (1940) and his associates.

UTILIZATION OF OXYGEN—RELATIONSHIP OF FREE OXYGEN TO GROWTH OF MICROORGANISMS

Some microorganisms will not grow in the absence of free oxygen, others will not develop in its presence, while still others can adapt themselves to either condition. An organism which requires free oxygen is termed *aerobic*, one whose growth is inhibited by oxygen is *anaerobic*, while one that grows under aerobic or anaerobic conditions is called *facultative*. Sometimes strict aerobes and strict anaerobes are termed obligate aerobes and obligate anaerobes. The fact that free oxygen may interfere with the growth of organisms has already been considered, and shown, in some cases, to be due to the absence of enzymes which would destroy the peroxides produced, particularly the absence of the enzyme catalase. The lines separating these groups based on oxygen relationships are often not well marked. Some organisms will grow neither in contact with oxygen at a pressure identical with that of the air nor in a medium totally devoid of oxygen. Such are sometimes termed *microaerophiles*. It is important to determine the concentration of oxygen necessary in such cases. Sometimes (particularly by those working with protozoa) the terms *oxybiontic* and *anoxybiontic* are used respectively for organisms which require and those that do not require free oxygen.

All organisms must secure energy for growth and movement through transformations of chemical compounds. This securing of energy from food is the result of oxidation no matter whether the organism is aerobic, anaerobic, or facultative.

Most cellular oxidations involve the removal of hydrogen from compounds rather than the addition of oxygen to compounds. Biological oxidations, in other words, are generally dehydrogenations. But it is a chemical axiom that whenever an oxidation occurs there is a simultaneous reduction. In other words, if cells oxidize their food by removing hydrogen from it, they must at the same time reduce something else by adding the hydrogen to the latter. Any substance which the cell oxidizes by removal of hydrogen is called a *hydrogen donator;* any substance to which the cell transfers the hydrogen is called a *hydrogen acceptor*. The cell secures the energy for growth and movement by this continuous process of hydrogen transfer. We are now able to redefine the term aerobe as applied to a cell. An aerobe is an organism which uses free oxygen as a hydrogen acceptor for the hydrogen taken from its foodstuffs in the process of oxidation. An obligate anaerobe is an organism which must use some substance other than oxygen as a hydrogen acceptor, in fact free oxygen is to be regarded as interfering with normal metabolism, that is, as poisonous. A facultative organism is one which can use a hydrogen acceptor other than oxygen and for which oxygen is not poisonous, some facultative forms may use oxygen, others do not. Several examples of hydrogen transfer will be given to illustrate the oxidative processes of the cells of microorganisms.

The acetic bacteria are good illustrations of aerobic cells which are able to obtain energy by the oxidation (dehydrogenation) of ethyl alcohol to acetic acid. It has been shown that this occurs in two steps: first, the oxidation of the alcohol to acetaldehyde and, second, the oxidation of the acetaldehyde to acetic acid. These transformations require the presence of free oxygen and water. The formation of the acetaldehyde may be illustrated as follows:

$$\underset{\text{Ethyl alcohol}}{CH_3{\cdot}CH_2OH} + \tfrac{1}{2}O_2 \rightarrow \underset{\text{Acetaldehyde}}{CH_3{\cdot}CHO} + H_2O$$

It is evident that in this case the alcohol has functioned as a hydrogen donator and the oxygen as a hydrogen acceptor. The next step is the oxidation of acetaldehyde (CH_3CHO) to acetic acid (CH_3COOH). It will be noted that the essential difference is apparently the presence of one more atom of oxygen in the acetic acid than in the acetaldehyde. This seems inconsistent with the statement above that biological oxidations are usually the result of removal of hydrogen rather than addition of oxygen. Apparently what happens is that acetaldehyde first combines with water to form acetaldehyde hydrate.

$$\underset{\text{Acetaldehyde}}{CH_3{\cdot}CHO + H_2O} \rightleftarrows \underset{\text{Acetaldehyde hydrate}}{CH_3{\cdot}CH(OH)_2}$$

It is from the latter compound that the hydrogen is removed, forming acetic acid.

$$\underset{\text{Acetaldehyde hydrate}}{CH_3{\cdot}CH(OH)_2} + \tfrac{1}{2}O_2 \rightarrow \underset{\text{Acetic acid}}{CH_3{\cdot}COOH} + H_2O$$

An *obligate anaerobe* was defined above as one that utilizes some substance other than oxygen as a hydrogen acceptor. Large numbers of such substances have been found to be utilized by different kinds of cells. Two types may be illustrated. In the first of these the food (the hydrogen donator) and the hydrogen acceptor are different substances. For example, bacteria are known which will oxidize ethyl alcohol and simultaneously reduce nitrates to nitrites using them as hydrogen acceptors. The reaction may be illustrated as follows:

$$\underset{\text{Ethanol}}{CH_3{\cdot}CH_2OH} + HNO_3 \rightarrow \underset{\text{Acetaldehyde}}{CH_3{\cdot}CHO} + HNO_2 + H_2O$$

In the second type of anaerobic utilization of food the hydrogen transfer occurs entirely within the food molecule itself. One carbon serves as a hydrogen donator and another as an acceptor. For example, yeast cells are known to change pyruvic acid to acetaldehyde and carbon dioxide.

$$\underset{\text{Pyruvic acid}}{CH_3{\cdot}CO{\cdot}COOH} \rightarrow \underset{\text{Acetaldehyde}}{CH_3{\cdot}CHO} + \underset{\text{Carbon dioxide}}{CO_2}$$

Intramolecular oxidation cannot be carried so far as is the case when the hydrogen acceptor is another molecule. This may be illustrated by comparing the amount of oxidation which dextrose may undergo when utilized by an aerobic bacterium on the one hand and by an anaerobic organism on the other. In aerobic transformation the dextrose is oxidized to carbon dioxide and water, and in anaerobic the dextrose may be transformed into lactic acid.

$$\underset{\text{Dextrose}}{CH_2OH(CHOH)_4CHO} + 6O_2 \rightarrow 6CO_2 + 6H_2O$$

In this change the carbon has all been completely oxidized, and all of the hydrogen has united with oxygen.

$$\underset{\text{Dextrose}}{CH_2OH(CHOH)_4CHO} \rightarrow \underset{\text{Lactic acid}}{2CH_3{\cdot}CH(OH){\cdot}COOH}$$

In this reaction some carbon atoms have been reduced, others have been oxidized. More energy is secured by the aerobic process than by the anaerobic, for in the first there is complete oxidation and in the second the oxidation is incomplete. One may get an approximation of the energy available to the two types of organisms, one aerobic and the other anaerobic, by measuring the amount of energy released as heat by each reaction. The ratio of heat production in the two cases is about 674:22. In other words, the transformation of dextrose into CO_2 and H_2O yields thirty-one times as much energy as the change into lactic acid.

Facultative bacteria are those which can grow under anaerobic conditions if certain compounds are present; with other combinations of nutrients growth can occur only in the presence of oxygen. For example, *Escherichia coli* in broth in the absence of sugar grows only aerobically; in the presence of a fermentable sugar the growth is equally good anaerobically.

The different requirements with respect to oxygen must be taken into consideration in cultivation of bacteria. Many devices are in use to regulate oxygen supply. For a discussion of these methods a standard laboratory manual should be consulted.

NITROGEN FIXATION

The third material which may be fixed by microorganisms is free nitrogen. The ability to utilize directly the free nitrogen of the air is not widely distributed among living cells. The economic importance of nitrogen fixation is greatest with reference to soil fertility.

The mechanism of nitrogen fixation has not yet been satisfactorily explained. It is currently assumed that an enzyme or more probably a series of enzymes catalyzes the reduction of gaseous nitrogen to ammonia (or possibly some other compound) which can be synthesized into amino acids.

CHANGES PRODUCED BY MICROORGANISMS IN NON-NITROGENOUS ORGANIC SUBSTANCES

Microorganisms bring about many changes in non-nitrogenous organic compounds. Most important are the alcoholic fermentations; the production of lactic and propionic acids from carbohydrates; the oxidation of alcohol to acetic acid; the production of butyl alcohol and butyric, citric, oxalic, and other acids from carbohydrates; the changes produced in cellulose, hemicellulose, pectins, and gums; the hydrolysis of fats; fermentations of the fatty acids; and various syntheses. These will be considered in order: first the production of ethyl alcohol.

Alcoholic Fermentation. *Types of Organisms Concerned in Alcoholic Fermentation.* Three groups of microorganisms, bacteria, yeasts, and molds, contain species which are capable of producing alcoholic fermentation of sugars in solution. However relatively few bacteria can bring about this change. The best known is *Pseudomonas lindneri* which may produce as much as 10 per cent alcohol in a suitable medium. None of them is of any considerable economic importance in the manufacture of alcohol. Certain of the molds, particularly those forms which simulate the yeasts in their growth in sugar solutions, are capable of inducing alcoholic fermentation, but they are most significant as producers of amylase with resultant ability to saccharify starch. Such are certain species of the genus *Aspergillus* (as *A. oryzae*) and of *Mucor* (particularly *M. rouxii*). These produce diastase (amylase) in large quantities. Under anaerobic conditions they are capable of forming small quantities of alcohol.

The most important organisms to be considered in alcoholic fermentations are the yeasts. Species belonging to many genera are able to produce alcohol, but most of those of commercial importance belong to the genus *Saccharomyces*. The differentiation of species is based largely upon the type of fermentation produced, the morphology of the cell, spore production, and the capacity of the organism to ferment various sugars. The sugars which are most commonly employed to test the ability of yeasts to produce alcoholic fermentation are glucose, maltose, sucrose, lactose, and raffinose. The yeasts are frequently classified with reference to their ability to ferment each of these sugars. Nearly all the forms can ferment glucose. A smaller number, but still a considerable proportion, can ferment maltose and sucrose, while a still smaller number, or relatively few, can ferment lactose or raffinose.

Enzymes Concerned in Alcoholic Fermentations. Several enzymes are indirectly and directly concerned with the production of alcohol. Certain *amylases* (*diastases*) are commonly responsible for the hydrolysis of starch into sugar upon which the yeast is to act. These amylases may originate in one of several ways. They may be produced by some seeds (as barley) in the process of germination, or by molds, or by bacteria. The former source is the one most commonly used in malting and brewing, while the second is sometimes used in the preparation of sugars to undergo fermentation in the production of commercial alcohol. Yeasts do *not* produce amylase. *Invertases* are also important, in as much as most yeasts ferment directly only the monosaccharides. Many of the yeasts produce some invertases, as *sucrase*, *maltase*, and even *lactase*, which are capable of inverting or hydrolyzing cane sugar, malt sugar, and milk sugar respectively. Some yeasts may attack disaccharides directly without inversion through catalysis by phosphorylases. In some cases molds or bacteria are in part responsible for the inversion of the disaccharides. *Zymase* is the name originally given to the enzyme supposed to be able to bring about the transformation of sugar into carbon dioxide and alcohol. It is now known to be a complex.

For many years alcoholic fermentation by yeasts was the stock example cited of a change brought about by an organized ferment, and the production of alcohol and carbon dioxide was conceived to be a part of the vital processes of the cell that could not be dissociated from life. In 1897 Buchner succeeded in extracting an enzyme (which he termed *zymase*) from the yeast cell and proved that it could bring about alcoholic fermentation in suitable sugar solutions. He mixed brewer's yeast with an equal weight of quartz sand and some infusorial earth. This was ground for some time in a mortar, a small amount of water added, and the juice forced out by the use of an hydraulic press, and clarified by filtration. This liquid was tested as to its zymase content by mixing with a suitable sugar solution. He found that in a short time carbon dioxide was evolved and alcohol formed. This yeast juice rapidly loses its power to produce changes, so that in a few days it is wholly inert. The literature treating on the subject of zymase, its preparation, and the changes which it brings about has become very extensive, and the phenomenon of alcoholic fermentation has been proved to be very much more complex than was originally supposed. Only a skeleton outline of the process can be given.

Zymase may be regarded as an enzyme mixture. It is believed that the complex changes involved in alcoholic fermentation are due to the presence in yeast of at least twelve different enzymes and five coenzymes. The first to act upon the simple sugars is phosphatase which catalyzes the union of phosphoric acid and hexose, to form a hexose phosphate. This compound is then broken down, and the sugar split to carbon compounds with three carbons in the chain. These are then probably transformed in normal alcoholic fermentation to pyruvic acid ($CH_3{\cdot}CO{\cdot}COOH$). Yeast (as a part of its zymase complex) contains the enzyme *carboxylase,* an oxidase. This enzyme attacks specifically keto acids, particularly those in which the keto group is in the alpha-position. It transforms pyruvic acid to acetaldehyde and CO_2.

$$\underset{\text{Pyruvic acid}}{CH_3{\cdot}CO{\cdot}COOH} \rightarrow \underset{\text{Acetaldehyde}}{CH_3{\cdot}CHO} + CO_2$$

This accounts for the production of the carbon dioxide of the alcoholic fermentation. The acetaldehyde is reduced to ethyl alcohol by acting as a hydrogen acceptor to one of the intermediate products.

$$\underset{\text{Acetaldehyde}}{CH_3{\cdot}CHO} + 2H \rightarrow \underset{\text{Ethyl alcohol}}{CH_3{\cdot}CH_2OH}$$

The whole reaction may be summarized as

$$\underset{\text{Glucose}}{C_6H_{12}O_6} \rightarrow \underset{\text{Ethyl alcohol}}{2CH_3{\cdot}CH_2OH} + 2CO_2$$

As will be noted later it is possible, by changes in the environment, to modify the course of the fermentation in various ways, and to secure considerable yields of other compounds such as glycerol.

The above account of the mechanisms of alcoholic fermentation is greatly abbreviated.[1] The subject is one that has been studied intensively in recent years, and a complete account of the various intermediate steps known in the process would require many pages. These studies have been of great significance in increasing our knowledge of general cellular physiology.

Alcoholic Fermentations of Economic Importance. The alcoholic fermentations of economic importance are the alcoholic fermentation of fruit juices, the alcoholic fermentation in brewing, the alcoholic fermentation of milk, the production of distilled liquors and of commercial alcohol, fermentation in the production of miscellaneous alcoholic beverages, such as pulque and ginger beer, coffee and cacao fermentations, purification of sugar mixtures, and the panary fermentations or production of carbon dioxide and alcohol in bread making.

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[1] That zymase is indeed a complex mixture of enzymes is illustrated by a listing of the steps in conversion of glucose to alcohol and CO_2 which have been suggested, but not in all cases confirmed.

1. $\text{Glucose} + \underset{\text{ATP}}{\text{adenosine triphosphate}} \xrightarrow{\text{Mg or Mn}} \underset{\text{Robison ester}}{\text{Glucose-6-phosphate}} + \underset{\text{ADP}}{\text{Adenosine diphosphate}}$
2. $\underset{\text{Robison ester}}{\text{Glucose-6-phosphate}} \xrightleftharpoons{\text{Isomerase}} \underset{\text{Neuberg ester}}{\text{Fructose-6-phosphate}}$
3. $\text{Fructose-6-phosphate} + \text{adenosine triphosphate} \xrightleftharpoons[]{\substack{\text{Phosphatase}\\ \text{Mg or Mn}}} \underset{\text{Harden-Young ester}}{\text{Fructose-1 : 6-diphosphate}} + \underset{\text{ADP}}{\text{adenosine diphosphate}}$
4. $\underset{\text{Harden-Young ester}}{\text{Fructose-1 : 6-diphosphate}} \xrightleftharpoons{\text{Aldolase}} \text{Dihydroxyacetone phosphate} + d\text{-3-glyceraldehyde phosphate}$

(4a). Dihydroxyacetone phosphate $\rightleftarrows$ *d*-3-glyceraldehyde phosphate

5. A series of changes occur involving inorganic phosphate and codehydrogenase II (diphosphopyridine nucleotide) and adenosine diphosphate with production of *d*-3-phosphoglyceric acid.
6. $d\text{-3-Phosphoglyceric acid} \xrightleftharpoons{\text{Phosphoglyceromutase}} d\text{-2-Phosphoglyceric acid}$
7. $d\text{-2 Phosphoglyceric acid} \xrightleftharpoons{\text{Enolase}} \text{Enol phosphopyruvic acid}$
8. $\text{Enol phosphopyruvic acid} + \underset{\text{ADP}}{\text{adenosine diphosphate}} \xrightleftharpoons{\text{Mg or Mn}} \text{Pyruvic acid} + \underset{\text{ATP}}{\text{Adenosine triphosphate}}$
9. $\text{Pyruvic acid} + \text{Diphosphothiamin (cocarboxylase)} \xrightleftharpoons{\text{Carboxylase}} \text{Acetaldehyde} + CO_2$
10. $\text{Acetaldehyde} + \underset{\text{(}H_2\text{DPN or reduced coenzyme I)}}{\text{reduced diphosphopyridine nucleotide}} \xrightleftharpoons{\substack{\text{Alcohol}\\ \text{dehydrogenase}}} \text{Ethanol} + \underset{\text{(DPN or coenzyme I)}}{\text{Diphosphopyridine nucleotide}}$

Alcoholic Fermentation of Fruit Juices. Most fruit juices containing sugars will undergo spontaneous alcoholic fermentation if expressed and allowed to stand for a time. The most important of the beverages produced in this way are wine, cider, and perry from the juice of the grape, the apple, and the pear respectively. The juices of many other fruits are occasionally used, such as those of raspberries, elderberries, blackberries, cherries, as well as other sugary juices from plants such as the palm and the agave.

The juice of the grape (*must*) contains considerable amounts of sugar, principally dextrose and fructose, sometimes as much as 25 per cent. In addition there is sufficient tartaric and other acids to inhibit the growth of most species of bacteria. The juices of the apple and of the pear usually contain between 15 and 20 per cent of sugar and sufficient acid (largely malic) and tannin to inhibit the growth of many undesirable organisms.

The microorganisms primarily responsible for the fermentation of these juices are yeasts, in most cases forms which occur upon the surface of the ripe fruits. These are present in sufficient quantities so that usually the addition of yeast is unnecessary for starting the fermentation. However, the kinds of yeasts naturally present may not always be those giving the best aroma, flavor, and quality of product. In many cases pure cultures of yeast are added to control the fermentation.

Yeasts may be demonstrated to be present upon the surface of ripe fruits by various means. It is not certainly known how they reach this point, as they do not occur usually upon the surface of the unripe or green fruits. It is probable that insects are largely responsible for their distribution. Yeasts generally occur in small numbers in most soils, and such yeasts gaining entrance through a puncture in the surface of the grape multiply and form a colony. Flies, bees, wasps, and other insects feeding upon this sugary material carry away considerable numbers of the cells, and in their flight from fruit to fruit serve to distribute them. False yeasts and molds are even more abundant than true yeasts upon the surface of these fruits, but under the conditions of the fermentation the true yeasts soon gain the ascendancy and prevent the development of other forms. Occasionally the false yeasts are present to the exclusion of others, and abnormal fermentations will then take place. The wine yeasts are generally included in the group formerly known as the species *Saccharomyces ellipsoideus*.

The fermentation of fruit juices generally requires that some oxygen be admitted, at least in the initial stages of the fermentation, to allow sufficiently rapid multiplication of the yeast cells. However, after fermentation has started, air is largely excluded. When fermentation is complete, it is frequently necessary to clarify by the addition of gelatin or albumin or at least by allowing the material to stand until the yeast cells and other suspended particles have settled out. These fermented fruit juices usually contain sufficient quantities of alcohol to inhibit the growth of most organisms, provided the oxygen of the air is excluded.

Beverages, such as wines and cider, produced from fruit juices may be spoiled by growth of certain undesirable organisms. Acetic acid bacteria will develop whenever there is sufficient exposure to oxygen. These grow usually upon the surface of the liquid, oxidizing the alcohol to acetic acid. Other organisms, including yeastlike fungi, may grow upon the surface of the solution in the presence of oxygen and oxidize the sugars, acids, and alcohol present to water and carbon dioxide. When alcohol is not developed in sufficient quantities, some kinds of bacteria may produce undesirable fermentation in the must or in the wine. Certain of the lactic acid bacteria may cause souring by conversion of sugar into lactic acid. Butyric acid bacteria, slime-producing bacteria, and molds may also destroy the product. In addition to the development of alcohol and carbon dioxide, there is more or less transformation of the protein compounds that may be in solution by proteolytic enzymes derived either from the fruit or from the development of yeasts. Changes in the color may be due to oxidases present. Undoubtedly the flavors characteristic of the wines of certain districts are largely due to the formation of byproducts of fermentation through enzymatic activity.

Alcoholic Fermentation in Brewing. The preparation of malt liquor or beer, with its varieties of porter, lager, and ale, may be divided into several stages, namely the processes of malting, mashing, fermenting, and aging.

Malt is usually prepared from barley, but other grains are sometimes used. The grain is soaked in water for a period of one to three days at a temperature low enough to discourage mold growth. It is then placed in vats or cylinders to germinate. It is regularly stirred or if in cylinders it is rotated to aerate and prevent heating. The germination proceeds until the sprout reaches half the length of the grain and is then stopped by drying with currents of warm air, the temperature being gradually raised as the drying progresses. When completely dry, the sprouts are removed by friction. The resulting grain is *malt*. The process of malting is designed essentially for the purpose of developing certain digestive enzymes, principally *diastase* (*amylase*), capable of converting starch into dextrins and sugar, certain *proteolytic enzymes,* and probably *cytase*. These enzymes are developed in the grain during the process of germination and function normally to convert the insoluble materials of the endosperm of the seed into soluble substances that can be utilized by the young plant as food. The process of germination is arrested in malting at the point where the maximum amount of enzyme has been produced and there is a minimum amount of change in other constituents; also before any considerable amount of the stored food has been utilized by the young plant.

In the process of *mashing,* the crushed or ground malt is mixed[2] with warm water and soaked for several hours and drained off. The amylase (diastase) rapidly converts the starch into dextrins and maltose, and the proteins of the grain are in part digested and dissolved by the proteolytic enzymes. This extract of malt is called *wort* or *beerwort*. It is removed from the solid materials and boiled to sterilize it, to aid in clarification, and in some cases to influence ultimate flavor. Hops are frequently added for the twofold purpose of contributing a characteristic flavor and aroma and to add certain antiseptic substances that aid in preservation and in prevention of abnormal fermentations.

The fermentation of the wort and its conversion into beer are initiated by the addition of yeasts. In modern practice this is usually accomplished by adding a relatively pure culture of some one of the varieties generally included under the species name *Saccharomyces cerevisiae*. Where pure culture methods are not employed, other yeasts may be active, such as *S. ellipsoideus* and the harmful *S. pastorianus*. Fermentation is carried out in vats. It proceeds with considerable rapidity for several days, then gradually subsides. The sugar and dextrins originally present in the wort are largely converted into carbon

[2] Usually ground corn (maize) or rice is also added to increase the starch content.

dioxide and alcohol. The beer is then placed in large casks, where it slowly continues to ferment, the yeast settles out, and the aging is complete in from a few weeks to several months.

Spoilage due to unwanted organisms may occur. Bacteria of the lactic acid type if not controlled may cause souring of the wort. Occasionally butyric acid bacteria may develop. Acetic acid bacteria cause deterioration of the beer when exposed to the air. Certain torulae and wild yeasts may gain entrance and produce disagreeable flavors, and slime-producing bacteria may form gummy dextrans and injure both consistency and flavor. Pasteurization is usually resorted to for the preservation of bottled beers.

Non-alcoholic malt beverages ("near beer") are prepared in the same general manner as beer, the alcoholic content being reduced to less than one-half per cent by distillation.

Alcoholic Fermentation of Milk. Milk usually undergoes lactic acid fermentation. There are few organisms capable of producing alcohol from lactose. Milk (more frequently cream) may undergo alcoholic fermentation following lactic fermentation. It is not usual for the lactic acid bacteria to ferment all of the lactose present in the milk or cream. The more slowly developing yeasts are not inhibited by the concentration of the lactic acid which stops the growth of the bacteria. They may then produce an alcoholic fermentation. Cans of cream, for example, shipped without refrigeration during hot weather may even blow off the sealed lid through the pressure of the accumulated gas. The development of the yeasts imparts an undesirable flavor and aroma, and may prove a serious difficulty in the manufacture of high-grade butter. Alcoholic beverages from milk are produced either through the activity of a lactose-fermenting yeast or by the addition of some sugar such as sucrose which can be fermented by the commoner types of yeasts. In all cases more or less lactic acid fermentation occurs simultaneously with the alcoholic, the inoculating material used and the temperature being the factors that determine the relative proportions of acid and alcohol.

A modified *koumiss* is sometimes prepared by the addition of cane sugar and yeast to milk; this is kept at blood heat until vigorous fermentation begins, then placed in tightly corked bottles and chilled. The lactose is utilized little or not at all in alcoholic fermentation, as the common bread yeasts do not ferment lactose. This fermentation results in the production of a beverage having a low content of alcohol (about 1 per cent) and acid (about 0.75 per cent), and one that is markedly effervescent. It is sometimes prescribed in the dietary of invalids and convalescents.

Koumiss is a beverage prepared originally by certain natives of southern Russia from mares' milk. Inoculation is made from a previous lot of koumiss. The fermentation in this case is due to the combined action of yeasts and bacteria, the former of the lactose-fermenting group.

Kefir is a beverage produced by the natives of the Caucasus. The milk is fermented as the result of inoculation with "kefir grains." These are dried zoogloeal masses of various sizes consisting of a mixture of yeasts and bacteria. These kefir grains are removed and preserved for future use after having initiated the fermentation. The product of the fermentation is a beverage containing about 1 per cent of acid and a small amount of alcohol. The yeast is called *Saccharomyces kefir*, differing from *S. cerevisiae* in its fermentive powers. *Leben* is a more acid beverage prepared in Arab countries, frequently from sheep's milk. The milk is heated nearly to the boiling point, cooled, and mixed with an inoculum from a lot of leben previously prepared. Alcohol is formed in small quantities; lactic acid is the principal fermentation product.

Fermented beverages prepared from milk will keep longer without becoming unfit for use than will the unfermented milk. In tropical countries particularly, the use of such beverages has long been practiced because of the difficulty of keeping milk fresh. The heating of the milk in preparation eliminates much of the danger of disease transfer.

Not only the sugars, but the proteins of the milk as well are usually somewhat changed in alcoholic fermentations, being partially digested.

Alcoholic Fermentation for Distilled Liquors and for Commercial Alcohol. Beverages of many types are produced by the distillation of fermented sugar solutions. *Brandy* is prepared by the distillation of fermented fruit juices, *rum* from fermented molasses or cane sugar syrups, *whisky* by the distillation of a fermented mash prepared from malted grain.

Commercial alcohol may be prepared by distillation from fermented sugar solutions of various kinds. The chief problem in its manufacture is to secure a satisfactory and cheap source of sugar.

The most important raw material for production of industrial alcohol in the United States is the molasses byproduct from sugar manufacture. Alcohol is also made from corn, potatoes, and surplus or low-quality grains. In recent years increasing quantities have been made synthetically from petroleum and natural gas.

Industrial alcohol has become a very important item in commerce. It is used as a solvent and as the starting point in the manufacture of numerous, probably hundreds of chemical products. Among these is the butadiene from which artificial rubber is produced. In many countries alcohol is used as fuel for internal combustion motors; it has marked antiknock properties when mixed with gasoline.

The starch of suitable raw materials may be converted into sugar for alcoholic fermentation in one of three ways. It may be hydrolyzed by the use of acids, by the addition of malt, or by the use of the enzymes produced by one of several molds. Conversion into sugar (saccharification) can be accomplished by heating the starchy material in a dilute solution of a suitable acid, such as sulfuric. The excess acid is neutralized before inoculation with the yeast. The use of malt does not differ in essentials from its use in the brewing industry. The third of the processes, that involving the use of certain molds deserves special mention. The first use of the *amylomyces* (starch fungus) process seems to have been in the Far East, where it was developed for the production of alcoholic beverages from rice. A leaven (*ragi*) is first prepared by mixing bits of sugar cane, root stalks, and rice meal. This is allowed to undergo spontaneous fermentation and becomes filled with the mycelium and eventually with the spores of certain molds, including two species of *Mucor*, (*M. rouxii* and *M. oryzae*). The pasty mass is made into cakes and dried, then is used to induce saccharification of rice. Rice is boiled until it becomes a pasty mass when it is mixed with the ragi or "Chinese yeast" and allowed to stand. The molds thus inoculated grow throughout the mass and by their production of amylase rapidly transform the starch into sugar. This old process has been adapted to modern techniques.

In the commercial amylomyces process the starchy material, usually corn, other grain, or potatoes is steamed for several hours under three to four atmospheres of pressure, cooled, and placed in a fermenting vat. A culture of a suitable *Mucor* or *Aspergillus* is introduced and thoroughly mixed with the contents of the vat. Sterile air is then bubbled through the mash. In the course of twenty-four hours, the entire mass will have been permeated by the mycelial threads of the fungus, and saccharification induced. The time of the completion of the conversion of the starch into sugar is determined by the use of the iodine test. The current of air is then stopped, yeast is added, and the sugar is converted into alcohol and carbon dioxide. When the content of alcohol has reached its maximum, the mash is distilled.

A more satisfactory adaptation of the amylomyces process has been made possible through the development of mold bran, which can be substituted quite directly for malt. The amylase-producing molds are grown on moist wheat bran until the maximum amylase content has been developed, when the fungus-matted material is dried and pulverized. This mold bran is then mixed with the starch substrate whereupon the amylase already formed functions rapidly to saccharify the starch.

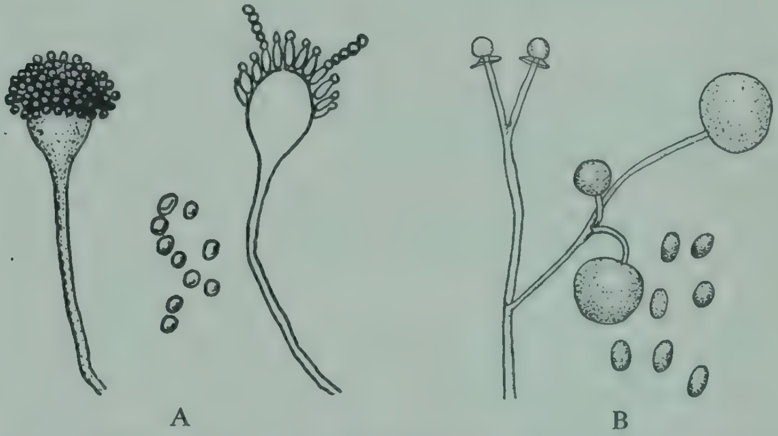


Fig. 22–1. Fungi important in saccharifying starch. A. *Aspergillus oryzae*, conidiophores and conidia. (Adapted from Wehmer.) B. *Mucor rouxii*, sporangiophores, sporangia, and spores. (Adapted from Wehmer.)

Attempts have been made to develop satisfactory methods for saccharifying sawdust and similar waste wood products in order that they may be used in alcoholic fermentation. While apparently technically feasible, the methods have yet to prove that they are profitable. However, commercial use is made of the process in at least one western plant in the United States.

Alcoholic Fermentation in Preparation of Other Beverages. Many beverages other than those already enumerated are produced by alcoholic fermentation of various saccharine solutions. Practically every country has its own peculiar product of this type. The juice of certain palms collected by cutting off the terminal bud yields *palm wine*. The sugary juice of the agave is used in Mexico for the production of *pulque*.

Ginger beer is prepared by adding ginger and sugar to water and inoculating with what is termed "ginger beer plant." This is an impure culture preserved in much the same manner as are grains of kefir for the fermentation of milk. Ginger beer is characteristically somewhat viscous as the result of the symbiotic growth of a yeast and a bacterium which form lactic acid and more or less gum. The organisms causing this fermentation are *Saccharomyces piriformis* and *Lactobacillus vermiformis*. Japanese *saké*, or *rice beer*, is of interest because saccharification of the rice used in its preparation preceding the fermentation by yeasts is brought about by *Aspergillus oryzae*, which has been mentioned above as sometimes useful in saccharification of starches for the production of commercial alcohol.

Pombe is produced in certain African countries by the fermentation of a malt prepared from millet. The fermentation is brought about by a member of the genus *Schizosaccharomyces*, *S. pombe*.

Mead is produced by the fermentation of honey diluted with water, usually with the addition of certain salts.

Alcoholic Fermentations in Food Processing and Manufacture. Alcoholic fermentation occurs during the processing of certain foods and feeds. In the preparation of *ensilage*, particularly from plants high in sugar, there may be some formation of alcohol, which is usually converted into acetic acid if oxygen has access. *Fermentation* of coffee is usually an essential part of the processing of this product. The coffee beans are removed mechanically from the cherry-like fruit in which they are produced. They are covered with a layer of gummy or mucilaginous material which contains sugar and acids. The seeds are placed in tanks and allowed to ferment spontaneously, when the pulp may be readily removed by washing and the coffee seeds dried. Whether or not the fermentation process modifies the flavor of the coffee is not clear. *Cacao* undergoes during its processing a somewhat similar fermentation. The seeds are embedded in an acid, sweet, mucilaginous pulp inside the cacao pod. The contents of the pods are scooped out and placed in suitable containers, when a spontaneous natural fermentation produced first by yeasts and then by bacteria occurs. The pulp can then be readily removed from the seeds by washing. Over-fermentation may produce an off flavor in the product.

Yeasts may be used in the purification of chemical products, particularly in the purification of sugars in mixtures. For example, milk sugar (lactose) may be hydrolyzed to a mixture of equal parts of glucose and galactose by use of acids and heat. The glucose may be removed by conversion to alcohol and CO_2 by growth of certain yeasts.

leaving the galactose unchanged to be purified further by crystallization.

Panary Fermentation. The series of changes which occur in the flour and other constituents of dough before baking into bread is termed *panary* fermentation. There is some conversion of starch into sugar (maltose), the production of gas, usually as a result of alcoholic fermentation brought about by yeasts, development of some acids, and minor changes in the flour proteins. The preparation of the various yeasts, or leavens, will first be considered, and this will be followed by a discussion of the changes they bring about in breadmaking and of certain abnormalities in bread due to undesirable microorganisms.

Preparation of Leaven. The leavens used for breadmaking may be divided into three groups: the relatively pure yeasts secured in the form of brewer's yeasts, compressed yeasts, or yeast cakes; the leavens propagated in the bakery or the home in solutions of various kinds (the "barms"); and the leaven that develops as a result of growth of organisms already present in the flour when mixed with water under appropriate conditions.

Brewer's yeast skimmed from the surface of the vats of fermenting wort is sometimes used in breadmaking. It spoils easily, must be kept cold, and at the best must be used within a short time after preparation. The compressed yeast most commonly used by bakers is a somewhat more concentrated product and may be kept in the cold for several days without deterioration. It is prepared in one of two ways, the so-called *old,* or *Vienna, process,* and the *new,* or *aeration, process.* In the *old process* a mash is prepared from grains such as rye, barley, maize, buckwheat, in some cases with the addition of potato. The grains are crushed, malt is added, and the mixture is soaked in water at 44°–50° C. This gives the optimum conditions for saccharification, and the diastase soon converts a large proportion of the starch into sugar. Frequently this is sterilized by heat to prevent growth of undesirable forms. It is acidified by the addition of acid mash, or by inoculation and fermentation with one of the lactic acid bacteria. It is then inoculated with yeast, usually a strain of *Saccharomyces cerevisiae*. An active fermentation ensues, and the yeast forms a heavy mat on the surface of the fermenting liquid. This is then removed and by means of a sieve is separated from any coarse particles. It is suspended in cold water (about 10° C.) and allowed to settle out; the water is drawn off and the yeast mass partially dried by pressure and forced into cakes. In the *new process* a wort is prepared much as in brewing by saccharification of various grains, or potatoes, by malt, followed by a filtration of the sugary extract. In some cases molasses is diluted and substituted for the wort. The acidity essential to prevent the growth of undesirable organisms is secured by inoculating with a lactic acid organism (as *Lactobacillus delbrueckii*) and holding at a relatively high temperature (50° C.) for a few hours. The liquid is cooled to 30° C., inoculated with a pure culture of yeast, and air bubbled through for a period of six to eight hours. Growth of the yeast is very rapid. It may be removed by centrifugation or by cooling and allowing it to settle to the bottom. It is washed and prepared as in the old process.

Starch, flour, or meal may be mixed with the yeast before it is pressed into cakes. The product may be dried. Yeast in a dried cake slowly loses its vitality; an old yeast cake, particularly one which has not been kept cool, may be wholly unable to initiate fermentation.

Leavens used for bread not only cause production of the essential gas, but also contribute to the flavor. The latter is due to the presence of microorganisms other than the yeast, particularly lactic acid and gas-producing bacteria. The continued propagation of all these organisms may be accomplished by holding a portion of the dough from one baking to be used in the next. Not infrequently, especially in England and Scotland, and to a less degree in the United States, these organisms have been propagated in special mixtures. Until recent years practically every locality and every successful bakery had its own particular formula for the preparation and propagation of the leaven, or barm. The usual constituents are water, potatoes, sugar, and hops. The hops are instrumental both in eliminating undesirable fermentations and in contributing a desirable flavor. These mixtures are ordinarily allowed to ferment spontaneously, although sometimes a little yeast is added to initiate the process. The composition of these barms unquestionably modifies materially the flavor and texture of the bread.

Flour usually contains of itself sufficient bacteria and yeasts so that when mixed with water gas will slowly develop. Undesirable organisms may be in part inhibited by the addition of salt and the control of the temperature. Such is the source of leaven in the so-called salt-rising or sour breads.

Fermentive Changes in Breadmaking. The essential in the preparation of all bread is to secure sufficient production of gas to cause the dough to rise, or become light. Generally, characteristic flavors and aromas develop also in consequence of this fermentation. The gas is usually the result of alcoholic fermentation of sugars by the yeasts present, but it is probable that in some cases bacteria also play an active part.

Flours and meals for the preparation of bread are usually made from wheat or rye, occasionally from maize or barley. They are all high in starch, and the first two contain a considerable proportion of protein, commonly designated as gluten. In addition there are, among other constituents, traces of sugar and some diastase. Flour is mixed with water to form a dough and for some types of bread a little sugar is also added. A small portion of the starch is converted into sugar by the action of the diastase. From this point the processes differ somewhat in the dough allowed to ferment spontaneously and that to which leaven of some kind is added.

To hasten fermentation various stimulants may be added. Flour normally contains enough β-amylase to change starch to sugar with sufficient rapidity, though sometimes small amounts of α-amylase are added to increase the rapidity of the conversions. Sugar, particularly maltose, may be included to insure prompt fermentation.

Baking destroys most of the organisms present and drives off much of the alcohol formed. The lactic acid is not volatile and remains.

Abnormal Fermentations of Bread. The most common abnormal fermentations in bread are: (1) undesirably high acidity, (2) ropiness, and (3) "bloody" bread.

Too high acidity may develop as the result of the growth of lactic bacteria for too long a period in the dough before baking, the bread becoming unpalatable as the result of the sourness.

Ropiness (rope, jack in the bread,) or slimy bread results from the growth, after baking, of certain highly resistant spore-producing bacteria. Several species have been described, closely related to *Bacillus subtilis,* quite probably variants of this species. The type of fermentation may occasion considerable trouble in a home, and in a bakery cause serious loss. These organisms seem to be present in most cases in the flour used; possibly in some instances they come from contamination of the utensils. They are probably present in most bread, but do not develop and cause rope except when there has been too little development of acid in the leavening process and the bread has been stored at a relatively high temperature. In other words, in order to prevent rapid deterioration of the bread after baking, it is necessary that a certain amount of acid be present in the dough. This is usually formed as a result of the growth of certain lactic acid species. Lactic or propionic acid may be added.

Some of the chromogenic bacteria may form colored spots or areas in the bread. *Serratia marcescens* (sometimes termed *Bacillus prodigiosus*) produces a red pigment. The effects of this organism have long been known. The red spots were interpreted before the development of modern science as spots of blood. An interesting history of the superstitions that have grown up about the phenomenon of bloody bread is given by Breed.

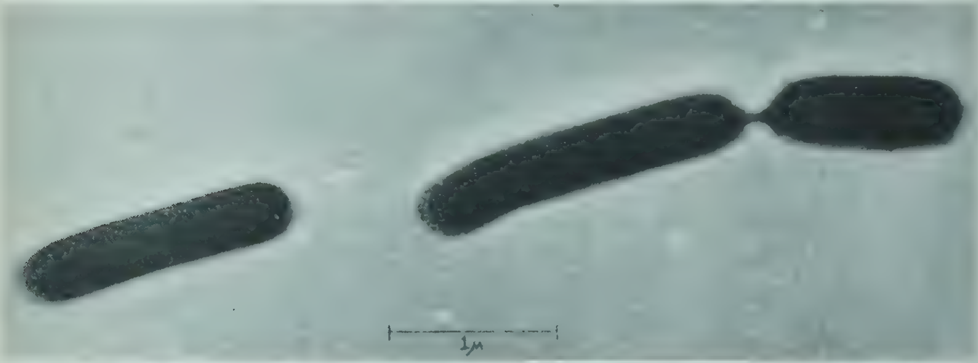


Fig. 22–2. *Lactobacillus acidophilus.* Electronograph. Note that one of the rods is in process of cell division and the protoplasm of the two cells is still connected by a strand termed a plasmodesmid (Courtesy of S. Mudd, K. Polevitsky, and T. F. Anderson and the *Archives of Pathology*.)

Glycerol Production. In the normal process of alcoholic fermentation small quantities of glycerol are formed. Usually 6 to 4 parts of glycerol are formed for each 100 parts of alcohol. The course of the alcoholic fermentation can be modified by the addition of bases or of sulfites so that the yield of alcohol is reduced and that of the glycerol increased. The overall reaction may be written

$$\underset{\text{Glucose}}{C_6H_{12}O_6} \rightarrow \underset{\text{Glycerol}}{CH_2OH{\cdot}CHOH{\cdot}CH_2OH} + CO_2 + \underset{\text{Acetaldehyde}}{CH_3{\cdot}CHO}$$

The sulfite combines with the acetaldehyde preventing its reduction to alcohol.

Glycerol is a compound of much significance in industry. Usually it is produced from fats and oils. When the latter are in short supply, as during World Wars I and II, the fermentation production of glycerol became quite important, and many millions of pounds were produced by this process.

Lactic Acid Fermentation. Lactic acid is one of the waste products formed during muscular activity in animals, and it arises as the principal product of the fermentation produced by many organisms. It is usually formed in milk and in fermented foods and feeds of the type of sauerkraut and ensilage.

Lactic acid is α-hydroxypropionic acid ($CH_3{\cdot}CHOH{\cdot}COOH$). Inasmuch as the carbon atom in the alpha-position is asymmetric, two lactic acids are known, the levolactic and dextrolactic acids, as well as the inactive form which is an equal mixture of the two. The kind of acid formed seems to depend upon the species of organism producing the fermentation, the sugar fermented, and the environmental conditions, including temperature. The overall equation for the transformation of sugar into lactic acid may be represented as

$$\underset{\text{Glucose}}{C_6H_{12}O_6} \rightarrow \underset{\text{Lactic acid}}{2CH_3{\cdot}CH(OH){\cdot}COOH}$$

Apparently, as in alcoholic fermentation, the transformation of sugar to lactic acid occurs in successive steps. Probably the steps are the same from glucose to the production of pyruvic acid as an intermediate. This compound is reduced to lactic acid.

$$\underset{\text{Pyruvic acid}}{CH_3{\cdot}CO{\cdot}COOH} + 2H \rightarrow \underset{\text{Lactic acid}}{CH_3{\cdot}CHOH{\cdot}COOH}$$

An alternative intermediate methyl glyoxal can also be transformed by some bacteria to lactic acid

$$\underset{\text{Methyl glyoxal}}{CH_3CO{\cdot}CHO} + H_2O \xrightarrow{\substack{\text{Glyoxalase}\\ \text{glutathione}}} \underset{\text{Lactic acid}}{CH_3{\cdot}CHOH{\cdot}COOH}$$

The bacteria most important in producing lactic acid may be placed in two groups, *homofermentative* and *heterofermentative*. In homofermentation, lactic acid is formed almost quantitatively from the sugar; in heterofermentation, additional compounds as carbon dioxide and acetic acid are produced. The mechanisms of the two types of lactic fermentation are somewhat different.

Homofermentative Lactic Microorganisms. Here are included principally members of the bacterial genera *Streptococcus* and *Lactobacillus* and the mold genus *Rhizopus*. Apparently with these organisms the intermediate pyruvic acid is transformed directly into lactic acid. This reaction also takes place in active muscles of the body. The hydrogen donator is reduced diphosphopyridine nucleotide (H_2DPN).

$$\underset{\text{Pyruvic acid}}{CH_3{\cdot}CO{\cdot}COOH} + H_2DPN \rightleftarrows \underset{\text{Lactic acid}}{CH_3{\cdot}CHOH{\cdot}COOH} + DPN$$

Many of the bacteria significant in milk and food fermentations belong in this group and will be considered in Chapter 24.

Heterofermentative Lactic Bacteria. Here are included many microorganisms which produce CO_2, acetic acid, some ethyl alcohol and glycerol, in addition to lactic acid. Some of those most studied belong to the genera *Lactobacillus* and *Leuconostoc*. Evidence developed by Nelson and Werkman indicates that the basic intermediate pyruvic acid is transformed in part into lactic acid, as with the homofermentative bacteria, and part into acetaldehyde and CO_2. The acetaldehyde is in part reduced to ethyl alcohol and in part oxidized to acetic acid.

$$\underset{\text{Pyruvic acid}}{CH_3{\cdot}CO{\cdot}COOH} + 2H \rightarrow \underset{\text{Lactic acid}}{CH_3{\cdot}CHOH{\cdot}COOH}$$

$$\underset{\text{Pyruvic acid}}{CH_3{\cdot}CO{\cdot}COOH} \rightarrow \underset{\text{Acetaldehyde}}{CH_3{\cdot}CHO} + CO_2$$

$$\underset{\text{Acetaldehyde}}{CH_3{\cdot}CHO} + 2H \rightarrow \underset{\text{Ethanol}}{CH_3{\cdot}CH_2OH}$$

$$\underset{\text{Acetaldehyde}}{CH_3{\cdot}CHO} + H_2O \rightarrow \underset{\text{Acetic acid}}{CH_3{\cdot}COOH} + 2H$$

Lactic fermentation of sugars is the source of the lactic acid so extensively used industrially, as in the tanning of leather, in dyeing, in foods in many ways, and in manufacture of plastics. In the United States about ten million pounds of lactic acid are produced annually. In commercial lactic acid fermentation by various processes starches may be saccharified as for alcohol manufacture, or raw materials containing such sugars as glucose, fructose, sucrose, or lactose may be used. Whey is a good substrate. The bacteria commonly used are members of the genera *Lactobacillus*, *Streptococcus*, and *Bacillus*. Under favorable conditions more than 90 per cent of the weight of the sugar fermented may be recovered as lactic acid. It is customary to use organisms which grow well at relatively high temperatures, 45° to 50° C. Usually calcium carbonate is added to neutralize the acid as formed. The fermented material is heated and filtered, the filtrate containing the calcium lactate is evaporated, and the lactic acid is separated by precipitation of the calcium with sulfuric acid.

The kind of lactic acid produced will depend on the kind of organisms used. Some form levo-, some dextro-, and some inactive lactic acid. Certain bacteria are able to transform levolactic to dextrolactic acid, and vice versa, with production of inactive acid.

Propionic Acid Fermentation. A group of bacteria, to which the generic name *Propionibacterium* has been given, are known to produce propionic acid ($CH_3 \cdot CH_2 \cdot COOH$) rather than lactic acid from carbohydrates. The species are closely related to *Lactobacillus,* but grow less readily under aerobic conditions. The fermentative changes involved in the production of propionic acid are probably, for most of the steps, similar to those in lactic fermentation, but the propionic bacteria have somewhat higher powers of reduction than do the lactic bacteria. The propionic acid bacteria usually also produce some gas (carbon dioxide), acetic acid, and succinic acid. They are important in the ripening of certain types of cheese.

Several mechanisms for the production of propionic acid have been suggested, perhaps more than one are used. One path postulated is the reduction of lactic acid.

$$\underset{\text{Lactic acid}}{CH_3 \cdot CHOH \cdot COOH} + 2H \rightarrow \underset{\text{Propionic acid}}{CH_3 \cdot CH_2 \cdot COOH} + H_2O$$

Possibly succinic acid may be decarboxylated to propionic acid.

$$\underset{\text{Succinic acid}}{HOOC \cdot CH_2 \cdot CH_2 \cdot COOH} \rightarrow \underset{\text{Propionic acid}}{CH_3 \cdot CH_2 \cdot COOH} + CO_2$$

While fermentative commercial production of propionic acid is technically feasible, there has not been enough industrial demand to warrant it.

Acetoin-Diacetyl Fermentation. The compounds acetoin ($CH_3 \cdot CHOH \cdot CO \cdot CH_3$) and diacetyl ($CH_3 \cdot CO \cdot CO \cdot CH_3$) are of interest in bacteriology for several reasons. The production of acetoin by some organisms and not by others is readily recognized by means of a color reaction, the Voges-Proskauer test, and may be used in their differential diagnosis. Acetoin is also converted by bacteria into diacetyl, the compound responsible for the characteristic aroma of butter. Acetoin is also convertible by bacteria into butane diol (2,3-butylene glycol). Several schemes have been outlined in an effort to explain these several reactions. Werkman and his associates concluded that with organisms producing acetoin (acetyl-methyl-carbinol) the production of pyruvic acid takes place in accordance with the scheme outlined for alcoholic fermentation, with further change to acetaldehyde. Two paths are possible leading to the formation of the acetoin, either,

$$\underset{\text{Acetaldehyde}}{2CH_3{\cdot}CHO} \rightarrow \underset{\text{Acetoin}}{CH_3{\cdot}CHOH{\cdot}CO{\cdot}CH_3}$$

or,

$$\underset{\text{Acetaldehyde}}{CH_3{\cdot}CHO} + \underset{\text{Pyruvic acid}}{CH_3{\cdot}CO{\cdot}COOH} \rightarrow \underset{\text{Acetoin}}{CH_3{\cdot}CHOH{\cdot}CO{\cdot}CH_3} + CO_2$$

The acetoin may be reduced by bacterial action.

$$\underset{\text{Acetoin}}{CH_3{\cdot}CHOH{\cdot}CO{\cdot}CH_3} + 2H \rightarrow \underset{\text{2,3-Butyleneglycol}}{CH_3{\cdot}CHOH{\cdot}CHOH{\cdot}CH_3}$$

Or it may be oxidized to diacetyl

$$\underset{\text{Acetoin}}{CH_3{\cdot}CHOH{\cdot}CO{\cdot}CH_3} \rightarrow \underset{\text{Diacetyl}}{CH_3{\cdot}CO{\cdot}CO{\cdot}CH_3} + 2H$$

The fact that 2,3-butylene glycol formed by bacteria can be readily converted into butadiene ($CH_2{:}CH{\cdot}CH{:}CH_2$) made the compound of much interest during World War II, as butadiene may be polymerized with other ingredients into synthetic rubber. The process was found to be feasible, but could not compete with the cheaper butadiene produced from natural gas or petroleum. About 45 per cent of the sugar fermented may under appropriate conditions be converted into 2,3-butylene glycol.

Production of diacetyl by fermentation is also feasible. It may be used to reinforce or to simulate butter aroma.

Acetic Acid Production. *Oxidation of Other Alcohols.* Acetic acid is most abundantly formed as the result of bacterial oxidation of ethyl alcohol, under aerobic conditions. The overall reaction is

$$\underset{\text{Alcohol}}{C_2H_5OH} + O_2 \rightarrow \underset{\text{Acetic acid}}{CH_3COOH} + H_2O$$

Acetic acid is also formed as one of the products of more or less complex anaerobic fermentations of carbohydrates, but in relatively smaller amounts. These latter are termed mixed or heterofermentations. Some organisms may produce small quantities of formic and acetic acid coincidentally with production of alcohol. The development of some acetic acid by propionic bacteria has already been noted. Of the organisms capable of producing acetic acid, the ones of greatest economic importance are the aerobic types that oxidize alcohol

These are the ones that are used in the manufacture of certain foods, as vinegar (Chapter 24).

Many species of aerobic acetic bacteria have been described, all belonging to the genus *Acetobacter*. They are closely related, differing in amount of acid produced, fermentative power, and in morphology. The oxidation of alcohol is believed to be due to a group of intracellular enzymes sometimes termed collectively *alcohol oxidase,* but they have never been separated from the bacterial cells.

There is reason to believe that the oxidation of ethanol takes place in two stages, the active enzyme being a dehydrogenase.

$$\underset{\text{Ethanol}}{CH_3{\cdot}CHOH} + \tfrac{1}{2}O_2 \rightarrow \underset{\text{Acetaldehyde}}{CH_3{\cdot}CHO} + H_2O$$

$$\underset{\text{Acetaldehyde}}{CH_3{\cdot}CHO} + \tfrac{1}{2}O_2 \rightarrow \underset{\text{Acetic acid}}{CH_3{\cdot}COOH}$$

Acetic acid is also produced synthetically and in the destructive distillation of wood.

Acetic acid prepared by acetification of alcohol may be readily purified and constitutes a part of the acid utilized industrially. Most of the acetic acid produced by fermentation is used as a condiment (vinegar).

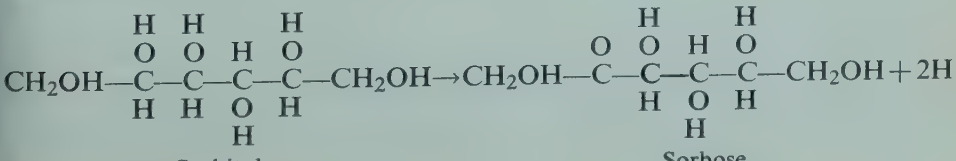
The ability of members of the genus *Acetobacter* to produce oxidation of alcohols is by no means limited to ethanol. Some of the changes produced have proved to be of some economic importance in industry. Among them may be noted the following.

Acetobacter xylinum and *A. suboxydans* can ferment glycerol with the production of a ketone, dihydroxyacetone.

$$\underset{\text{Glycerol}}{CH_2OH{\cdot}CHOH{\cdot}CH_2OH} \rightarrow \underset{\text{Dihydroxyacetone}}{CH_2OH{\cdot}CO{\cdot}CH_2OH} + 2H$$

It has been found more economical to produce this compound by the use of bacteria than by synthetic methods.

The compound *sorbitol,* a polyatomic alcohol found in certain fruits, is converted into the carbohydrate *sorbose* by the activity of various acetic bacteria, particularly *A. xylinum.* The reaction may be illustrated



$$CH_2OH-\underset{H}{\overset{\substack{H\\O}}{C}}-\underset{H}{\overset{\substack{H\\O}}{C}}-\underset{\substack{O\\H}}{\overset{H}{C}}-\underset{H}{\overset{\substack{H\\O}}{C}}-CH_2OH \rightarrow CH_2OH-\overset{O}{C}-\underset{H}{\overset{\substack{H\\O}}{C}}-\underset{\substack{O\\H}}{\overset{H}{C}}-\underset{H}{\overset{\substack{H\\O}}{C}}-CH_2OH+2H$$

Sorbitol Sorbose

This reaction is of importance because sorbose is the starting point in the making of ascorbic acid (vitamin C) and may be produced commercially by use of this bacterial oxidation.

The acetic bacteria are able also to form gluconic acid, and the several keto derivatives of this acid. Gluconic acid will be discussed separately as it is usually produced as the result of a mold activity.

Still another oxidation is the production of *d*-tartaric acid COOH·CHOH·CHOH·COOH from 5-keto-gluconic acid. This acid is usually produced as a byproduct in the fermentation of grape juice, but may be manufactured by use of *Acetobacter*.

Butyric Fermentation. The formation of butyric acid is one of the undesirable fermentations encountered in the decomposition of carbohydrates under anaerobic conditions by certain bacteria. This acid is usually only one of the products of the activity of the butyric acid bacteria, hence it is difficult to determine with certainty the reactions involved. The overall reaction for butyric acid production is as follows:

$$\underset{\text{Dextrose}}{C_6H_{12}O_6} \rightarrow \underset{\text{Butyric acid}}{C_3H_7COOH} + 2CO_2 + 2H_2$$

Because of the powerful and disagreeable odor produced, this type of fermentation is of economic importance in food processing industries and in food and feed spoilage.

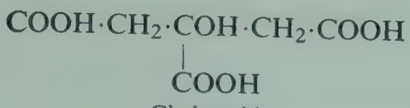
The bacteria chiefly responsible for production of butyric acid are closely related, resembling each other morphologically and culturally, and differing only in minor physiological characters. They are widely distributed in the soil. All are spore-bearing bacilli, usually clostridia, developing only under anaerobic conditions.

The mechanism of butyric acid fermentation has not been satisfactorily worked out. The first steps in the dissimilation of the sugar are probably the same as in alcoholic fermentation. It is assumed that acetaldehyde formed may be condensed.

$$\underset{\text{Acetaldehyde}}{CH_3{\cdot}CHO + CH_3{\cdot}CHO} \rightarrow \underset{\text{Acetaldol}}{CH_3{\cdot}CHOH{\cdot}CH_2{\cdot}CHO}$$

and the acetaldol both oxidized and reduced to form butyric acid: $CH_3{\cdot}CH_2{\cdot}CH_2{\cdot}COOH$.

Citric Acid Production. Citric acid is produced in considerable quantities by the commercial transformation of sugar by several species of molds. A great amount of work has been devoted to attempts to determine the course of the changes which occur in transforming a straight chain 6-carbon sugar into a branched chain 6-carbon compound.



$$\mathrm{COOH{\cdot}CH_2{\cdot}\underset{\substack{|\\ \mathrm{COOH}}}{C}OH{\cdot}CH_2{\cdot}COOH}$$

Citric acid

It is possible that the citric acid is produced as the result of the formation of shorter-chained intermediate products which combine to form the citric acid. Many schemes have been postulated, but none is at present verified. The process is surprisingly efficient; the citric acid produced may be as much as 85 per cent of the weight of the sugar used.

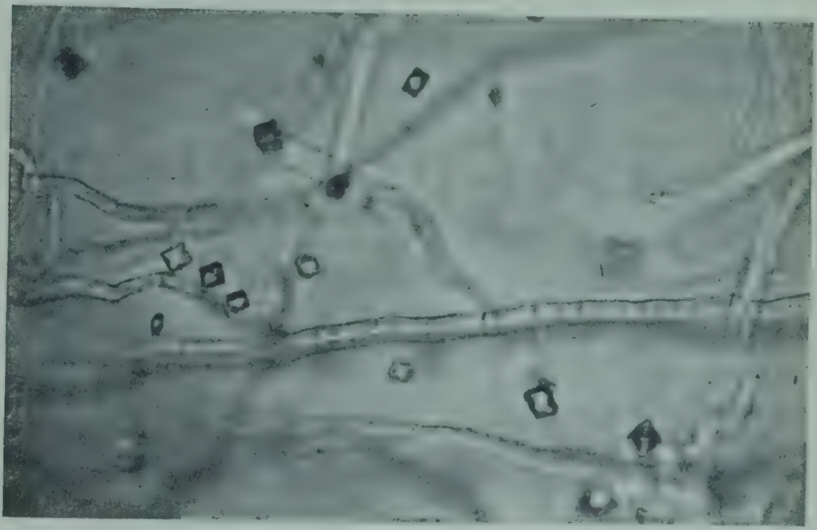


Fig. 22–3. Crystals of calcium oxalate produced by *Aspergillus* growing in a sugar agar medium.

The organisms best known as producers of citric acid are several species of the mold genera *Aspergillus* and *Penicillium*. These molds have been found in acid fruits. They can produce citric acid from sugar to a concentration of about 8 per cent, and much greater if neutralized by lime. Citric acid is the principal acid of the lemon and orange and has an extensive industrial use. Citric acid is produced on a commercial scale by the use of these molds and is able to compete from the standpoint of cost with the acid extracted from fruits. More than 15 million pounds of citric acid are produced annually by fermentation.

Oxalic Acid Production. Certain molds, particularly of the genus *Aspergillus* (as *A. niger*), produce appreciable amounts of oxalic acid when growing in a suitable sugar solution. When the acid as formed is neutralized by the addition of chalk, half the calculated theoretical yield from cane sugar may be obtained. The oxalic acid production by molds has not been utilized commercially and is of little economic significance.

The overall reaction may be written:

$$\underset{\text{Sugar}}{C_6H_{12}O_6} + 4\tfrac{1}{2}O_2 \rightarrow \underset{\text{Oxalic acid}}{3COOH{\cdot}COOH} + 3H_2O$$

The intermediate steps have not been positively identified. The following reactions have been postulated, among others:

$$\underset{\text{Acetic acid}}{CH_3{\cdot}COOH} + O_2 \rightarrow \underset{\text{Glyoxalic acid}}{CHO{\cdot}COOH} + H_2O$$

$$\underset{\text{Glyoxalic acid}}{CHO{\cdot}COOH} + \tfrac{1}{2}O_2 \rightarrow \underset{\text{Oxalic acid}}{COOH{\cdot}COOH}$$

Gluconic Acid Production. Since the discovery that certain species of bacteria of the genus *Acetobacter* could oxidize glucose with the production of gluconic acid, numerous species of molds, particularly species of the genera *Aspergillus* and *Penicillium*, have been found able, likewise, to form this compound. The production of gluconic acid by a fermentative process is of some commercial significance. Of the various molds studied, *Penicillium chrysogenum* has given the most satisfactory yields and results. The reaction appears to be

$$\underset{\text{Glucose}}{CH_2OH{\cdot}(CHOH)_4{\cdot}CHO} + \tfrac{1}{2}O_2 \rightarrow \underset{\text{Gluconic acid}}{CH_2OH{\cdot}(CHOH)_4{\cdot}COOH}$$

Certain other molds and bacteria are known to be able to convert other sugars to the corresponding acids, as mannose to mannonic acid, galactose to galactonic acid, xylose to xylonic acid, and arabinose to arabonic acid.

The enzyme responsible for catalyzing the reaction can be secured from the mycelium of the fungus. It is a glucose oxidase. The production of gluconic acid commercially requires adequate aeration. This has been accomplished by growing the culture in a rotating drum. Calcium carbonate is added to the sugar medium to neutralize the acid as it is formed. Gluconic acid amounting to 95 per cent of the sugar used has been secured.

The calcium salt of gluconic acid has been extensively utilized in human and in veterinary medicine for supplying calcium deficiencies in children, and in the treatment of milk fever in the cow.

Oxidation of Organic Acids by Microorganisms. Many microorganisms, principally molds and non-sporing yeasts, can oxidize organic acids in the presence of an abundant supply of oxygen to carbon dioxide and water. This accounts in large measure for the gradual loss in strength of vinegar and for the disappearance of lactic acid in sour milk, sauerkraut, etc. By such changes the inhibiting effect of the acid is removed and putrefactive organisms may then develop.

Acetone and Butyl Alcohol Fermentation. Several bacteria, particularly species of the genus *Bacillus* (*Aerobacillus*), produce acetone, $(CH_3)_2CO$, in the fermentation of carbohydrates such as starch. Certain other obligate anaerobic forms of the genus *Clostridium* are able to produce butyl alcohol (C_4H_9OH) from starch. The commercial production of each of these compounds has developed into industries of considerable significance.

Two distinct groups of bacteria produce acetone in the course of the fermentations which they induce. The members of the genus *Bacillus* belonging to the subgenus *Aerobacillus* form acetone and ethyl alcohol, and certain species of the anaerobic genus *Clostridium* which produce acetone and butyl alcohol. The importance of acetone in the manufacture of explosives led, during World War I, to rather intensive study of the first reaction as a possible source. *Bacillus macerans* was found to ferment many carbohydrates with the production of acetone, ethyl alcohol, acetic acid, carbon dioxide, and hydrogen. It is probable that the acetic acid is transformed in part into acetone:

$$\underset{\text{Acetic acid}}{2CH_3\cdot COOH} \rightarrow \underset{\text{Acetone}}{CH_3\cdot CO\cdot CH_3} + CO_2 + H_2O$$

Utilization of the second group of organisms (*Clostridium*) in production of acetone and butyl alcohol proved more practicable, and these compounds have been manufactured in large quantities by the fermentation process. It was found that the butanol produced had a ready market in industry, particularly in the manufacture and use of plastics. The organism usually used is a strain of *Clostridium acetobutylicum*. Corn and molasses are most often employed as raw materials, although many other carbohydrates, as potato starch, may be utilized. The products of fermentation are removed by distillation.

The mechanism of the various reactions involved have not been adequately clarified. For the production of acetone it has been suggested that the acetic acid formed is transformed into acetoacetic acid and this in turn into acetone.

$$\underset{\text{Acetic acid}}{2CH_3\cdot COOH} \rightarrow \underset{\text{Acetoacetic acid}}{CH_3\cdot CO\cdot CH_2\cdot COOH} + CO_2$$

$$\underset{\text{Acetoacetic acid}}{CH_3\cdot CO\cdot CH_2\cdot COOH} \rightarrow \underset{\text{Acetone}}{CH_3\cdot CO\cdot CH_3} + CO_2$$

It is not improbable that the butyl alcohol is derived from butyric acid, but the process of its production is not clear. The overall reaction may be written:

$$\underset{\text{Butyric acid}}{CH_3\cdot CH_2\cdot CH_2\cdot COOH} + 4H \rightarrow \underset{\text{Butyl alcohol}}{CH_3\cdot CH_2\cdot CH_2\cdot CH_2OH} + H_2O$$

Transformation of Cellulose and Hemicelluloses. Cellulose $(C_6H_{10}O_5)_n$ is a component of most plant cells, and is universal in the higher or green plants as the principal constituent of the cell wall. It is the most abundant carbohydrate in nature. The changes which it may undergo as a result of fermentation are therefore of considerable importance. It can be broken down into dextrose by heating with an acid. The hemicelluloses are also abundant in plants. They are much more readily hydrolyzed than is cellulose. Most of the hemicelluloses when heated with acids break down or hydrolyze into sugars other than dextrose. The hemicellulose of some seeds, such as the date, is hydrolyzed to mannose; many legumes yield galactose; still other seeds and plants, xylose or arabinose.

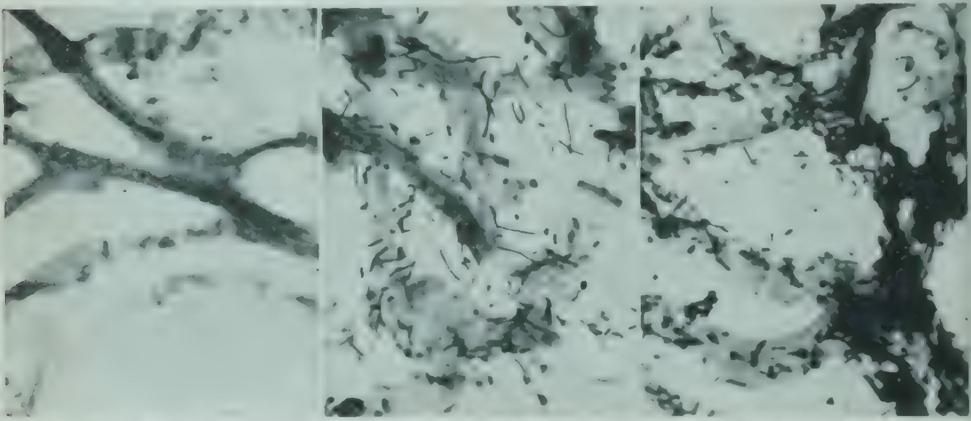


Fig. 22–4. Stages in the decomposition of the hyphae of the fungus *Pleurotus* through the activity of bacteria. A. Relatively few bacteria, the hyphae little changed. B. The hyphae somewhat swollen, showing incipient disintegration and numerous bacteria. C. Hyphae largely destroyed and replaced by bacteria.

Many species of bacteria and molds can ferment and utilize the hemicelluloses, fewer forms can utilize the true celluloses. The change may in some cases consist in an initial hydrolysis of the polysaccharide into its sugar by the enzyme cytase or cellulase, for example, the disaccharide cellobiose may be formed by the hydrolysis of cellulose. The production of this enzyme by microorganisms may be demonstrated by growing a suitable form on agar containing very finely divided particles of cellulose in suspension. The medium surrounding the colonies of the organism will clear as a result of the secretion of the enzyme and the consequent solution of the cellulose.

The organisms responsible for the decomposition of cellulose are in two main groups, the anaerobic forms and the aerobic; the former are all bacteria, and the latter include bacteria, molds, and higher fungi.

Although cellulose is the most abundant of the carbohydrates and the decomposition or digestion of the cellulose by microorganisms is a matter of common observation, surprisingly little is known of the processes involved. It seems evident that enzymes of the phosphorylase group are responsible for the synthesis of these complex compounds. It is therefore probable that these enzymes are produced by organisms and are in part responsible for the breakdown of these polysaccharides. When once sugar has been formed from the cellulose, the changes that may take place are of course extremely varied. Organisms that are able to attack cellulose are abundant in nature. We shall find them important in the changes which take place in the soil; they are responsible for the digestion of cellulose in sewage and wastes; they are important in the ripening of composts; they are important geologically through their activity in the formation of peats and coal; they may be used for the preparation of many chemicals by fermentation. Among the compounds that have been identified in the various fermentations of cellulose are butyric acid, acetic acid, ethyl alcohol, methane, carbon dioxide, and hydrogen.

Fermentation of Starch and Inulin. The transformation of starch by microorganisms may be simple hydrolysis (a conversion into sugar), followed by the fermentation of the sugar or the starch molecule may be directly attacked, without evidence of sugar formation. Starch is a polysaccharide of the empirical formula $(C_6H_{10}O_5)_n$. It is one of the most important of food constituents, forming the major portion of the carbohydrate diet of man and most animals.

Many molds and bacteria are known to saccharify starch. This is accomplished by the action of the hydrolytic extracellular enzyme diastase (amylase). Starch is first converted into a series of polysaccharides termed *dextrins*, differing from starch in being soluble in water. The dextrins are of several types, probably representing grades of complexity. The one first formed stains red with iodine and is called erythrodextrin. The simpler forms give no iodine reaction and are called achroodextrin. These dextrins are then hydrolyzed to maltose. Some maltose is also formed in the earlier stages of the hydrolysis, along with the dextrins. Many organisms carry the change a step farther by the hydrolysis of maltose to dextrose. The saccharification of starch for the production of alcohol by *Mucor rouxii* and *Aspergillus oryzae* has already been discussed under the heading of alcoholic fermentation. As a result of this hydrolysis sugars are formed which may be fermented in various ways by a great variety of organisms.

Starches and the closely related glycogen are also both synthesized and broken down by the enzyme phosphorylase produced by many microorganisms. It is possible therefore for such organisms to attack starch directly without any preliminary hydrolysis to sugar.

Inulin has the same empirical formula as starch, but hydrolyzes to fructose instead of glucose. It is found in many plants, particularly members of the family *Compositae* such as the dahlia and the Jerusalem artichoke. It is fermented by many bacteria and is one of the carbohydrates usually tested to determine the fermentative capacity of a microorganism.

Fermentation of Pectins. The pectins are complex polysaccharides yielding sugars and uronic acids on hydrolysis. Neutral pectins are found in the cell walls and the cell sap of many plants. The calcium salt of a related compound, pectic acid, constitutes in part the binding material between the cells and fibers of higher plants (except where modified into wood); it is the middle lamella. A cross section of the stem of any of those plants, when properly stained, will show that the cellulose walls of the cells are not in contact with each other, but are separated by a pectic layer.

Pectins are even more abundant in the softer plant tissues, such as in fruits and certain vegetables.

Many organisms producing disease in plants or decay in plant tissues have been found to secrete an enzyme, *pectinase*, which hydrolyzes the pectins of the middle lamella, loosening the union of the plant cells or fibers. Several bacteria, for example, have been described which produce so-called soft rots in vegetables, bulbs, etc. As the organism advances, the tissues before it soften, owing to the digestive action of the pectinase, and the cells are easily separable from each other. The same type of enzyme is produced by certain pathogenic and decay-producing fungi.

Practical use is made of this property of microorganisms in the separation of plant fibers in the process of retting flax and hemp. Linen fibers are the bast fibers of the flax plant. In cross section of a stem of the flax, these bast fibers are seen as white, many-sided angular cells, with a thick cell wall of cellulose, the cell cavity practically obliterated. These occur in groups, each cell separated from its neighbor by a middle lamella. In longitudinal section the bast cells are found to be long, pointed at the tips, and lying parallel to each other. The production of linen is a problem in separating these bundles of bast fibers from the stems. This is accomplished by the process called *retting* ("rotting"). Two principal methods are used: submergence in water and exposure to moisture, as dew and rain; the so-called water and dew retting processes, respectively. In the former the flax is tied into bundles and submerged in ponds or streams and weighted. Bacteria resembling *Clostridium butyricum* morphologically, but specifically different, have been obtained in pure culture and proved to be capable of producing all the necessary changes. Among those described are *Clostridium pectinovorum* and *C. felsineum*. The changes produced consist primarily of a softening of the middle lamella, a breaking apart of the cells of the plant tissues, and a weakening of the bond between the bundles of bast fibers. The retting must be interrupted at the right stage or the bacteria capable of digesting cellulose will attack the flax fibers and eventually destroy them. These bundles are then dried, and the bast fibers are removed and separated from each other by mechanical means. In the dew-retting process the flax is allowed to lie upon the ground, with the moisture supplied by the dew and rain, until various molds, and possibly bacteria, have destroyed the pectins and permit the removal of the fibers. The retting of hemp is carried out in essentially the same manner as described for flax. Investigations on utilization of pure cultures of the organisms responsible for retting have been successful, but such methods have not come into common use.

Transformation of Gums. Gums are compounds closely related to cellulose and the hemicelluloses; all swell considerably in water, some go into solution, and when heated with acids they hydrolyze into simple carbohydrates (mostly reducing sugars), and organic acids. Gum arabic, for example, is decomposed into arabinose and galactose, cherry gum into arabinose, gum tragacanth to arabinose, xylose, and fucose. These gums are not easily attacked by microorganisms, but in nature they slowly undergo decomposition in much the same manner as does cellulose. Several organisms have been described as capable of digesting gelose, the principal constituent of the unusually resistant agar-agar prepared from certain seaweeds and extensively used as an inert material for the solidification of media. Several species of bacteria have been described capable of liquefying agar; apparently in the seas they play much the same role as do the cellulose bacteria in the soil.

Fermentation of Fats. Fats are glycerides of fatty acids. The general formula is $C_3H_5{\cdot}R_3$, where R represents a fatty acid radical. The three fats most common are palmitin $(C_{15}H_{31}COO)_3C_3H_5$, stearin $(C_{17}H_{35}COO)_3C_3H_5$, and olein $(C_{17}H_{33}COO)_3C_3H_5$. Three stages or types of changes are to be considered in the fermentation of fats: the hydrolysis of fats into glycerol and fatty acids, the fermentation of the glycerol, and the fermentation of the fatty acids thus formed.

The hydrolysis of a fat (stearin) may be illustrated:

$$\underset{\text{Stearin}}{(C_{17}H_{35}COO)_3C_3H_5} + 3H_2O \rightarrow \underset{\text{Glycerol}}{C_3H_5(OH)_3} + \underset{\text{Stearic acid}}{3C_{17}H_{35}COOH}$$

This change is produced by the fat-digesting or lipolytic enzyme *lipase*. The enzyme is formed by many microorganisms, principally bacteria and molds. The ease with which fats can be largely freed from water explains the fact that they can be preserved as food for a considerable period of time. In the presence of moisture, however, and particularly in the presence of other organic matter, fats are actively hydrolyzed by many organisms. Kruse lists eighteen species capable of producing this change. They are important as the common cause of spoiling of many fat foods, although it is probable that in most cases this simple hydrolytic cleavage is not directly responsible for the sum total of rancidity developed or for disagreeable odors and flavors.

Fermentation of Glycerol. The glycerol formed as one of the products of hydrolysis of fats undergoes various changes as a result of the activity of microorganisms. *Escherichia coli,* among others, converts it into alcohol and formic acid.

$$\underset{\text{Glycerol}}{C_3H_5(OH)_3} \rightarrow \underset{\text{Alcohol}}{C_2H_5OH} + \underset{\text{Formic acid}}{HCOOH}$$

Clostridium orthobutylicum and many other clostridia produce butyric acid, carbon dioxide, and hydrogen. The overall reaction may be represented as

$$\underset{\text{Glycerol}}{2C_3H_5(OH)_3} \rightarrow \underset{\text{Butyric acid}}{C_3H_7COOH} + 2CO_2 + 2H_2$$

although the several steps in the reaction are undoubtedly much more complex. Inasmuch as these bacteria are very commonly present in the soil and produce resistant spores, they ordinarily withstand the process of pasteurization and are not uncommon in milk and in butter. The organisms may develop within the water globules of the butter. These droplets contain small quantities of milk sugar, and proteins; which enable bacteria to start growth under favorable conditions and, by means of the hydrolyzing enzymes or lipase they produce, cause the freeing of glycerol, which they then ferment with the production of butyric acid and related compounds. Butter fat normally contains a small percentage of butyrin, and the hydrolysis of this fat is responsible for the liberation of some of the butyric acid. The use of salt in the preparation of butter insures that the water globules shall be practically saturated solutions of sodium chloride. Under these conditions the hydrolytic bacteria do not develop rapidly. The salt, in other words, acts as a preservative.

Fermentation of Fatty Acid. The fatty acids produced as a result of hydrolysis of the fats may also be changed by certain microorganisms. Very few species have been described, however, which are capable of transforming these compounds. The salts of the simpler fatty acids, such as formic, acetic, propionic, valerianic, and butyric, can be utilized by many species of molds and a few bacteria; usually, however, only in the presence of oxygen.

Transformation of Paraffins. Organisms have been described which can oxidize paraffins and related compounds. These changes take place very slowly, as the paraffins are among the most resistant of the organic compounds known.

CHANGES PRODUCED BY ORGANISMS IN ORGANIC NITROGENOUS COMPOUNDS

General Discussion. The proteins and related compounds are complex organic substances containing carbon, hydrogen, oxygen, nitrogen, usually sulfur, and frequently phosphorus or iron. The molecules of ordinary proteins are of great size. All are alike in that by a series of hydrolytic cleavages they may be converted into simpler and simpler compounds, the *essential* ultimate products in every case being α-amino acids. This fact in the hands of Emil Fischer and his associates led to a fair understanding of the manner in which proteins are constituted. The amino acids can be caused to combine; for example, two molecules of glycocoll, $CH_2\cdot(NH_2)\cdot COOH$, can, by a series of transformations, be caused ultimately to unite, with the elimination of water. Disregarding the intermediate products the process may be represented as follows:

$$\underset{\text{Glycocoll}}{NH_2CH_2COOH} + NH_2CH_2COOH \rightarrow \underset{\text{Glycyl glycine}}{NH_2CH_2CONHCH_2COOH} + H_2O$$

Such a compound is termed a *peptide*. In a similar manner other amino acids may be caused to combine to form peptides (dipeptides, tripeptides, etc.). It will be noted that the peptides are also amino acids; hence they, too, can combine to form more complex compounds, termed *polypeptides*. A polypeptide has been formed synthetically in the manner indicated above by the union of eighteen molecules of amino acids, having the molecular weight of 1213. There is probably no real distinction to be drawn between a polypeptide and a peptone. Decomposition of proteins shows that by the process of hydrolysis they are broken down into these same products. We can then conceive of the protein molecule as being built of constituent amino acids (sometimes called the "building stones" of the protein), these united into groups called peptides, and these peptides into still larger groups called peptones, and these in turn into proteins. How many kinds of proteins are there? As will be noted in a later discussion of serology, differentiation among the many kinds of proteins to be found in an animal or plant is possible. It has been suggested that there must be hundreds of millions, perhaps thousands of millions of kinds. Brand and associates (1945) illustrated well the complexity of a single protein, β-lacto-globulin which they studied with care. They showed that the molecular weight closely approximated 42,000. The empirical formula given is $C_{1864}H_{3012}N_{428}S_{16}O_{576}$. It apparently has about 370 amino acid residues with 366 polypeptide bonds. Upon hydrolysis the numbers of the several constituent amino acids determined were as follows: glycine, 8; alanine, 29; valine, 21; leucine, 50; isoleucine, 27; proline, 15; phenylalanine, 9; cysteine, 8; methionine, 9; tryptophan, 4; arginine, 7; histidine, 4; lysine, 33; glutamine, 24; serine, 20; threonine, 21; tyrosine, 9.

The decomposition of proteins by bacteria as well as by chemical means is to be considered as a successive breaking down of the protein into compounds having a lower and lower molecular weight, eventually to amino acids. By many organisms the transformation is carried still further, and the nitrogen appears as ammonia. In addition to these hydrolytic cleavage products formed by the growth of organisms, however, there are many nitrogenous compounds produced that are in part synthetic. It is frequently difficult to differentiate these from the breakdown products.

Microorganisms may be classed in three groups on the basis of their ability to attack proteins. Some organisms are wholly unable to utilize proteins as nutrients; some of these can utilize peptones or amino acids, but some can secure nitrogen for development only from inorganic compounds. A second group may be able to grow upon proteins, but do not bring about any marked degree of digestion or profound modification in them. Such are many of the pathogenic bacteria, among them the organisms causing pneumonia, tuberculosis, and diphtheria. The third group includes these organisms that take an active part in the breaking down of organic nitrogenous matter in nature. Some of these liquefy solid or coagulated proteins, rendering them soluble by conversion into proteoses and peptones. This process may be termed *proteolysis* (formation of peptones). The same, or other, organisms may transform the peptones into amino acids, and these into ammonia.

Proteins cannot serve directly for the nutrition of microorganisms. They must first be transformed into soluble compounds capable of diffusing through plant membranes, particularly the ectoplast of the cell. This change must come about as the result of the activity of extracellular enzymes. But living bacterial cells go much farther and bring about many oxidative and reductive changes in the products of the hydrolytic cleavage of the proteins. Some of the compounds produced may be malodorous, and the change is termed *putrefactive*.

Among the many compounds thus formed are ammonia and amines, carbon dioxide, acids of the aliphatic and aromatic series, hydrogen sulfide, mercaptans, methane, phenol, skatol, indole, and others. Organisms important in bringing about decomposition of proteins may be divided (Kruse) into four distinct groups: the strict anaerobes, the facultative anaerobes, the aerobic bacteria, and certain molds. These will be considered in turn, the principal organisms of each group being described together with the most important compounds formed as a result of the decompositions they bring about.

Anaerobic Putrefactive Group of Bacteria. True putrefaction, that is, the disintegration of proteins with the production of various foul-smelling compounds, is generally ascribed to a group of anaerobic bacteria. Among them are certain disease-producing forms, such as those causing blackleg in cattle (*Clostridium feseri*) and malignant edema (*C. novyi*). The list of saprophytic forms is now a long one, but they seem to be for the most part closely related. *C. putrificum* and *C. perfringens* are among the most common and important.

Clostridium putrificum (*C. lentoputrescens*) is common in laboratory dust and in feces. There is good reason for believing that it (or some one of the closely related species) is the commonest cause of putrefaction of meat and other proteins. It is a slender rod with rounded ends, 5 to 6 μ in length, single or in chains. Spores are produced at the ends of the rods. They are greater in diameter than the mother cell and give a drumstick appearance to the rod. The organism grows readily in artificial media. It may be isolated by inoculating a mixture of egg white and water with soil, cheese, or fecal material and heating to 80° C. for ten minutes. This destroys all non-spore-forming organisms. *C. putrificum* can grow in such a medium; most other bacteria cannot develop in native proteins. An intense putrefactive odor soon appears, due to the development of hydrogen sulfide (H_2S) and methyl mercaptan (CH_3SH). The solid protein is liquefied; peptones, amino acids, and related compounds being formed. The butyric acid and ammonia developed also contribute to the odor. It should be emphasized that this organism is the type of the few bacteria that can decompose native proteins such as egg albumin, fibrin, and serum albumin under anaerobic conditions and in the absence of carbohydrates. It is the putrefactive bacillus *par excellence*. It is believed to be of importance in the intestines, particularly the colon, under certain diseased or abnormal conditions, as the cause of intestinal putrefaction.

Clostridium welchii (*C. perfringens*) may be taken as representative of a large group of organisms that can cause active decomposition of proteins in the presence of carbohydrates. It is common in soil and has been repeatedly isolated from excreta. It is a large gram-positive rod, 1 μ by 3 to 6 μ, single or in short chains. It is non-motile (most of the related forms are motile). Spores are not readily produced except on the surface of blood serum under anaerobic conditions. This is not true of all related forms, as some develop spores abundantly in culture media. The spores are equatorial in position and the cells become swollen or spindle-shaped. Capsules may sometimes be observed. *C. welchii* may be isolated from soil by inoculating sterile milk, covered with paraffin oil to exclude the air, with an infusion of rich soil and heating to 80° C. This does not destroy the spores of this organism, but will kill the usual lactic acid forms. After incubation for twenty-four hours at blood heat, the milk will be found to be coagulated and developing gas. The casein is digested. Organisms of this type are probably quite as common and important in the decomposition of proteins as is the *C. putrificum*. Carbon dioxide and hydrogen are formed most abundantly from sugar, but are also produced though more slowly from proteins. Butyric acid, hydrogen sulfide, mercaptans, indole, and other foul-smelling compounds may be formed. Gaseous anaerobic putrefaction may be generally regarded as brought about by this organism or a closely related species.

Group of Facultative Anaerobic Proteolytic Bacteria. This is commonly designated as the proteus group of bacteria, due to the proteus-like or ameboid character of the colonies which these organisms form on gelatin. Several members of the group have been described, the most important being *Proteus vulgaris* and *Proteus mirabilis*. The first may be regarded as typical of the group. All are facultative anaerobes causing active decomposition of proteins.

Proteus vulgaris has been repeatedly isolated from decaying meat. It is a motile bacillus 0.6 μ by 1.2 to 4 μ. It stains readily, is gram-negative, and does *not* form spores. It grows readily on culture media. On agar and gelatin it forms thin, irregular colonies; liquefaction occurs in the latter medium. Development is best at room temperature. It produces acid and gas from dextrose and sucrose, but not from lactose. Casein is liquefied, but it is somewhat doubtful whether this organism can digest such proteins as fibrin and egg albumin under anaerobic conditions. Indole and other malodorous compounds may be formed in sugar-free media.

Aerobic Proteolytic Bacteria. Certain of the spirilla are able to decompose some native proteins and related compounds such as gelatin. Among these is the Asiatic cholera organism. Such forms probably do not play any very important part in nature in the decomposition of nitrogenous compounds. Far more important is the *Bacillus subtilis*, or hay bacillus, group of bacilli. All these organisms are able to decompose proteins under aerobic conditions, frequently without the development of disagreeable odors characteristic of putrefaction. The principal representative of the group are *B. subtilis* or the hay bacillus and *B. cereus* in its several varieties. Many other species have been described. They are everywhere abundant in the surface soil and probably constitute the most important group of decay-producing organisms in nature. They are particularly active in the production of ammonia. *B. cereus* may be used to illustrate the characters of the hay bacillus group.

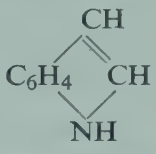
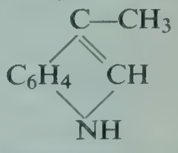
Bacillus cereus is a large rod, usually occurring in long chains, and commonly with truncated ends. It is very sluggishly motile in young cultures, non-motile in older cultures. It produces spores readily. These are equatorial in position and do not usually cause a marked enlargement of the cell. The cells stain readily and are gram-positive. The organism grows well upon culture media. The colonies on agar are quite characteristic. They are exceedingly irregular and spreading, and, when examined under the low power of the microscope, are found to consist, at least on the margins, of masses of long filaments like curled hair. In gelatin stabs these filaments radiate from the line of stab, giving a characteristic inverted fir tree appearance. Gelatin is liquefied. Milk is first coagulated by a rennet-like enzyme and the casein afterwards digested. Blood serum and some other proteins are also liquefied. Peptones and amino acids are formed, with a large proportion of the nitrogen eventually appearing as ammonia. Occasionally aromatic compounds such as skatole and indole may be formed, as also some of the fatty acids, such as acetic, butyric, and valerianic. These organisms are important in nature in decomposing nitrogenous compounds with the formation of ammonia. They are essential in agriculture for the maintenance of soil fertility.

Another group of aerobic proteolytic bacteria of considerable importance is that of the green fluorescent bacilli. The chief representative of this group is *Pseudomonas fluorescens.* This is an organism not uncommon is water, and is sometimes present in soils. It is an actively motile rod with polar flagella, stains readily, is gram-negative, and does not produce spores. When grown on suitable nutrient media, a green fluorescent pigment diffuses through the medium. Gelatin is liquefied, and other related compounds are digested, and ammonia is produced.

Molds Producing Proteolysis. Many molds have been described that can proteolyze native proteins and related compounds. Among these are species of *Penicillium, Aspergillus, Cephalothecium,* and *Mucor.* A long series of degradation products resulting from their activity have been described. Ammonia is developed in considerable quantities by some forms. At least two species of *Penicillium* will be noted as of considerable importance in the ripening of cheeses.

Putrefactive Substances Having Disagreeable Odors and Flavors. A complete discussion of the compounds capable of affecting the flavor or aroma that may be formed in nitrogenous food would include a discussion of practically all decomposition products formed. Only a few distinctive products among those most commonly found will be noted.

Butyric acid (C_3H_7COOH) and acetic acid (CH_3COOH) are commonly formed in protein decomposition. In the putrefaction of such foods as meats, it is possible that these may originate from the fats or carbohydrates present rather than from the proteins. Some putrefactive bacteria, however, do form butyric acid from the native proteins.

Indole  and methyl indole, or skatole  are commonly formed by many organisms from native proteins or peptones in the absence of carbohydrates. These have an intensely disagreeable fecal odor. They are developed from the amino acid tryptophan.

Among the most disagreeable of the odors developed are those of the mercaptans, particularly methyl mercaptan (CH_3SH). These and certain related compounds containing sulfur are the common causes of the worst odors attendant upon putrefaction. Hydrogen sulfide is also commonly formed, as in rotten eggs.

Ammonia is sometimes evolved in sufficient quantities to be distinctly noticeable. Some of the compounds listed among the ptomaines are also malodorous, as trimethylamine ($(CH_3)_3N$), often detectable in the brine used for preserving herrings.

Production of Amines. Certain microorganisms when growing under anaerobic conditions may attack amino acids, eliminating carbon dioxide, thereby changing the compound from an amino acid to an amine. This process is termed *decarboxylation*. It may be illustrated by the following reaction:

$$\underset{\text{Alanine}}{CH_3CHNH_2COOH} \rightarrow \underset{\text{Ethylamine}}{CH_3CH_2NH_2} + CO_2$$

Amines produced as a result of the action of microorganisms have been termed *ptomaines*. Some of the amines thus developed are malodorous. A few are distinctly poisonous, as for example, the histamine formed by the decarboxylation of the amino acid called histidine. Among the amines most commonly produced by microorganisms is *trimethylamine* $(CH_3)_3N$. This gives a characteristic odor to herring brine and decaying fish. It is produced by certain bacteria in pure cultures. It is not highly poisonous. Other ptomaines which have been isolated are *putrescine* (tetramethylenediamine $NH_2C_4H_8NH_2$), *neurine* $(CH_3)_3C_2H_3NOH$, and *cadaverine* (pentamethylenediamine $NH_2C_5H_{10}NH_2$). The second is very poisonous. It should be noted that apparently food poisoning is but rarely due to the presence of ptomaines. Most of the cases of so-called ptomaine poisonings are in reality either infections with specific bacteria or are the result of eating foods containing true toxins or endotoxins produced by specific bacteria, and are not primarily the result of a decomposed protein and the presence of a true ptomaine.

There has been much speculation as to the origin of petroleum. Evidence seems to point to its being produced through decomposition of organic matter, such as the bodies of the algae and animalcules that fall to the ocean floor. It has been suggested that the paraffines of petroleum might result from a single decarboxylatine of fatty acids.

$$\underset{\text{Fatty acid}}{CH_3(CH_2)_nCOOH} \rightarrow \underset{\text{Hydrocarbon}}{CH_3(CH_2)_{n-1}CH_3} + CO_2$$

That this change can be effectuated by a species of *Desulfovibrio* isolated from oil sands of oil wells has been shown by Jankowski and ZoBell.

CHEMICAL SYNTHESES BROUGHT ABOUT BY MICROORGANISMS

Living cells, as we have seen, literally bring about myriads of chemical reactions which are primarily analytical in nature. But such degradation of compounds is a means to an end and not an end in itself. In part the analytic changes are of a nature such that in various coupled reactions the energy available as a result of the analytic changes makes possible the synthesis of a multitude of complex organic compounds characteristic of the several cells. The analytic action also serves to produce the simple building blocks such as amino acids and the several monosaccharides which may be directly polymerized into complex compounds. Obviously many kinds of compounds are formed, so many that it is not practicable to enumerate even all the *groups* of synthetic compounds that have been thus elaborated. A few of these groups are sufficiently important to be considered. They are: the mucins, gums, and capsular materials, the toxins and related compounds, the pigments, and antibiotics. These last are so significant as to warrant treatment in a separate chapter (Chapter 23).

Synthesis of Mucins and Gums. *Capsular Substances.* The cell walls of most bacteria and molds are nitrogenous, but in some cases they give the characteristic reactions of cellulose. The nitrogenous wall is frequently termed *chitinous,* indicating that in a composition it is like the *chitin* which constitutes the outer covering of insects and related forms. It is sometimes much thickened, the outer portion being a swollen capsule even reaching a diameter several times that of the cell itself. This capsular material may produce certain changes in the physical structure or consistency of the medium. In later discussions involving immunity and serology it will be noted that these capsules are sometimes significant in helping to explain the attacking power or virulence of certain disease-producing bacteria. Some of them give reactions which may be useful in disease diagnosis. Chemically these materials may be divided into two groups, the mucins and the carbohydrate polymers, or gums.

The mucins are the slimes produced by certain organisms when growing in a protein- or peptone-rich medium such as milk or beef broth. They may be identified by precipitation with alcohol, followed by re-solution in water, repeating the process several times. The mucins give most of the characteristic protein reactions. When heated with acids they acquire the property of reducing Fehling's solution, due to the fact that they contain a carbohydrate radical. They evidently are complex nitrogenous compounds breaking up readily into proteins and carbohydrates. Bacteria producing mucins have been described as one cause of slimy milk, but it is probable that in most cases this is due to the development of gum-forming bacteria of the type to be next described.

The gums or gumlike carbohydrates synthesized by microorganisms are of many types, depending perhaps in part on the carbohydrate present in the medium. Mannans and dextrans, polymeric anhydrides of mannose and dextrose respectively, are among the commonest. Galactans and levulans also have been noted. These gumlike substances either swell considerably in water or dissolve in it more or less completely. They are precipitated by alcohol. When heated with acid they break down into uronic acids and into simple sugars which give the Fehling reaction. Chemically they are closely related to the vegetable gums.

Gummy or slimy fermentation has frequently been a source of trouble in the manufacture of cane sugar. The organism responsible is *Leuconostoc mesenteroides* which produces jelly-like masses, sometimes of considerable size. The organism grows readily in media without sugar but does not then produce a capsule. In solutions of cane sugar, however, it forms thick capsules which fuse into a gelatinous mass. This gum of the capsule has the empirical formula $(C_6H_{10}O_5)_n$ and when heated with acids is hydrolyzed to dextrose. It is therefore a dextran. Slime production in paper pulp during its manufacture may cause the appearance of translucent spots in the paper, so-called fisheyes.

Wine sometimes undergoes a viscous fermentation, being changed to an oily liquid that can be drawn out into filaments. Micrococci such as *Micrococcus viscosus* have been described as the cause.

Beerwort and beer not infrequently undergo slimy fermentation. This is particularly true where pure culture methods are not utilized in the fermentation process. A number of bacteria have been described as capable of bringing about this change.

Various plant infusions, unless sterilized or kept from fermenting by the addition of preservatives, may undergo gummy fermentation. This is true of infusions of digitalis, senega, extracts of various other herbs, and sugary solutions of all kinds. These fermentations are particularly troublesome to the pharmacist.

Slimy fermentation of milk has been described by many investigators as caused by several distinct species of bacteria. Some of them have been described as producing mucin. Most of them, however, produce galactans or dextrans. *Streptococcus hollandicus* is utilized for the production of *"lang Wei"* or ropy whey and milk, the latter being used in the preparation of Edam cheese. Similar organisms have been described from the Scandinavian *"lang Milch."* Some species of *Aerobacter* are capable of forming an unusual amount of slime. Other organisms, micrococci, streptococci, and bacilli have also been described as important slime producers. The ability to produce capsules is somewhat evanescent, as a culture seemingly without reason may acquire or lose this property. It is sometimes found in creamery practice that the pure culture used as starter after transfer to the milk may acquire the power of forming slime. Occasionally the milk from the udder of one cow in a herd will contain these slime-producing organisms and the milk of the herd will become slimy under favorable conditions. In other cases sufficient care is not used in the cleaning and sterilization of the milking utensils; in still other cases the water supply has been found to be involved. Some of the bacteria which produce slime in milk also produce considerable quantities of acid, others are gas producers, others seem to be more or less inert, and still others bring about the development of decidedly disagreeable flavors. The ropy milk of Holland and the Scandinavian countries already mentioned is commonly used as a beverage. The peculiar flavor is probably not altogether due to the slime-producing organism but to the associative action of several species.

The theory has been advanced by Grieg-Smith and others that many of the gums supposed to be physiological secretions of plants, such as the gums produced on certain acacias, gum tragacanth produced on a leguminous plant (*Astragalus*) and gum arabic, are in reality due to the growth of certain species of bacteria in the sap which exudes from wounds in these plants. Pure cultures of some of these organisms have been found to produce very similar gums when grown in suitable nutrient media in the laboratory.

Synthesis of Other Nitrogenous Compounds. Microorganisms may synthesize nitrogen into complex compounds of several types, the most important being the protoplasm of the organism, enzymes, mucins, probably toxins and endotoxins and hormones, vitamins and growth accelerants. The protoplasm, mucins, and enzymes have already been noted.

Toxins are to be sharply differentiated from the poisonous ptomaines discussed above. They are the products of synthetic activity; their chemical composition is unknown; they can be tested only by biological means (animal injection); they are easily destroyed by heat and when injected in repeated non-lethal doses into a suitable animal stimulate the production of an antitoxin which is capable of neutralizing the toxin. The toxins will be considered at greater length in a later chapter on immunity.

Endotoxins are also directly or indirectly products of synthetic action. Like toxins they are poisonous, but unlike them are frequently retained within the cell of the microorganism. Antitoxins are not formed for endotoxins when these are injected into the bodies of animals.

Microorganisms manufacture a great variety of compounds usually grouped together as vitamins and growth accelerants. For example, certain vitamins essential to the health and development of animals cannot be synthesized by the animals themselves. They may occur in the plant tissues used as food, but in some cases, as with ruminants, sufficient amounts are not available from this source. The needed vitamins are produced by the activity of microorganisms within the alimentary tract. This synthesis will be considered later under the topic of digestion in ruminants. In some cases substances thus produced may also prove stimulating to other organisms. For example, the organism *Mycobacterium paratuberculosis;* the cause of Johnes disease in cattle, requires for its growth the presence in the medium of the growth products of some other related species of acid-fast bacteria.

Many synthetic vitamins and accessory factors are produced by microorganisms. Some of these may be developed in sufficient quantities so that commercial production is feasible. For example certain species of the yeast genus *Candida* (as *C. guilliermondii* and *C. flareri*) may produce from 100 to 325 gamma of riboflavin per milliliter in a suitable medium. Another of special interest is the vitamin B_{12} complex which is at least a part of the so-called animal protein factor. It has long been known that certain animals and birds, particularly swine and the domestic fowl, seemed to require some animal protein in the diet. It was found possible to extract from liver this animal protein factor and by feeding this material greatly to increase the efficiency of the utilization of plant proteins such as soybean meal. Then it was discovered that this material is produced also by certain microorganisms, such as a strain of *Streptomyces aureofaciens*. It may be commercially possible to produce the material cheaply enough to make it practicable to include it in the protein rations of animals. Brink and his associates (1949) have determined some of the characteristics of the compound. It has a molecular weight of about 1490, and contains carbon, hydrogen, nitrogen, phosphorus, and cobalt. It is not a peptide, because upon hydrolysis no α-amino acids are formed.

Pigments. Many yeasts, molds, and bacteria are said to be *chromogenic,* that is, they produce *pigments,* or coloring matter. A few bacteria contain within their bodies a pigment, *bacteriopurpurin.* It is a red-purple coloring matter characteristic of organisms able to assimilate carbon dioxide by the aid of sunlight. In many others the pigments are to be regarded as secondary waste product having no very apparent physiological function. A pigment called *cytochrome* listed under enzymes has been found to be present in most living cells and to function in cell respiration. Cells possessing pigments in the cell walls may perhaps in some cases be protected from light rays which might be injurious and which are absorbed by the pigment. In most cases, the pigment produced is insoluble in water and remains within the cell or cell wall. The mycelium or spores of one whole family of molds, the *Dematiaceae,* are brown or fuscous; the spores of many other molds are of bright colors such as green, yellow, orange, and red. Bacteria producing pigments of this type are abundant, particularly the red and yellow forms, though in addition species are known that duplicate practically every color of the spectrum. Some of these are of economic importance, such as *Serratia marcescens* which produces red spots and blotches on carbohydrate foods and is responsible for the so-called bloody bread. Blue milk, red milk, and red spots on cheese are also due to bacteria. Pink, brown, and black yeasts are not uncommon.

Pigments are usually classified by their solubility in various agents, such as water, ether, alcohol, and chloroform. Chemically they belong to very different groups. Some are *lipochromes,* or pigments commonly found in fats and soluble in fat solvents; they are usually red, yellow, or orange. Others are quite insoluble in the common fat solvents. The chemical composition of a few pigments has been determined (as of pyocyanine), but for many it is unknown.

In many cases the color produced is determined by the character of the medium in which the organism is grown. Some pigments produced by microorganisms change color with changes in acidity and may even be used as hydrogen ion indicators.

Most organisms produce pigments only in the presence of free oxygen, although there are some chromogenic anaerobes. The property of pigment production may be lost by an organism, and it may not be recovered even by cultivation under conditions most favorable for its development.

BIOCHEMICAL UTILIZATION OF MICROORGANISMS

The high degree of specificity shown by certain microorganisms in their utilization of nutrients and selectivity for the organic compounds which they decompose or ferment has made the use of bacteria, yeasts, molds, and even protozoa of increasing significance in analytic organic chemistry and biochemistry. These organisms can be used to differentiate and even to determine quantitatively minute concentrations of certain substances such as vitamins and likewise to identify and measure certain closely related compounds as they occur in mixtures. An area of biochemistry is rapidly developing in which microorganisms play a leading role in chemical determinations. Following are examples of a few of the specific uses that have thus been made.

The products of hydrolysis of certain complex polysaccharides found in the stems and wood of plants are found to contain mixtures of sugars difficult to separate and determine quantitatively. Microorganisms, particularly yeasts, have been found useful. For example, the hemicelluloses of agricultural residues (Auernheimer and associates, 1948) may be hydrolyzed and a mixture of the pentose sugars *d*-xylose and L-arabinose secured. When this mixture is added to a suitable medium the yeast *Hansenula suaveolens* will remove (utilize) all of the *d*-xylose, while the yeast *Candida guilliermondii* will utilize both sugars completely. By determining the copper-reducing values of samples fermented by each organism, the percentage of each sugar may be calculated. This procedure replaces the older chemical tests which were difficult and time-consuming.

It is known that the structure of the molecule of the amylose fraction of starch differs from that of the amylopectin. Waxy starch, practically pure amylopectin, is produced by certain strains of maize. The products of hydrolysis by pancreatic amylase gives rise to dextrins, maltose, and some glucose. Information of value with reference to the molecular structure may be secured by determination of the proportion of the products of hydrolysis. Organisms which are able to ferment glucose but not maltose may be used, also organisms which can ferment both sugars. Estimates of amounts of the sugars present may be made by determination of the amounts of the products of fermentation.

Qualitative and even quantitative estimates of amounts of a considerable number of compounds may be made by use of a microorganism which requires a definite nutrient for growth but is quite unable to synthesize it. Such essential substances have been termed growth factors. Some species of microorganisms naturally require one or more of these growth factors; in other cases mutants of microorganisms have been secured which are unable to grow in the absence of certain growth factors. Various bacteria, as members of the genus *Lactobacillus,* molds such as *Neurospora,* and yeasts have been used. A culture medium is prepared containing everything necessary for the growth of the test organism except the growth factor for which the determination is to be made. The medium is distributed into suitable culture tubes, and different quantities of the material under test are placed in the several tubes. The tubes are then inoculated with the test organism. Checks are run using as amendments to the several tubes varying quantities of the compound sought. Suitable comparisons of the amounts of growth of the test organisms will make possible the estimation of the amount of the compound in the sample under study.

Among the compounds that have been found to be growth factors, the following have been reported: Aneurin, β-alanine, biotin, glutamine, inositol, nicotinic acid, pantothenic acid, para-amino-benzoic acid, pimelic acid, pyridine nucleotides, pyridoxine, riboflavin, folic acid, vitamin B_{12}, purines, and pyrimidines.

An organism which has proved most useful in the quantitative determination of various materials as well as in the sequence of events in chain reactions which occur in cells is *Neurospora crassa* and its numerous mutants. It was found by Beadle and his associates that by suitable irradiation and by other treatments mutations may be induced. Not infrequently a mutant will be found that fails to synthesize some essential food compound as could the parent strain. For convenience in terminology such a mutant strain may be named from the compound it no longer can synthesize, but which is essential to its growth. Usually the suffix "less" is added. For example, choline is an essential nutrient for *Neurospora,* and the "wild" strains of the fungus are able to synthesize it for use. A mutant strain which is *cholineless* has been found. The amount of choline present in a medium otherwise adequate may be estimated with a high degree of accuracy by determining the amount of growth produced. Another mutant, *leucineless*, has been used for the determination of leucine in the mixture of amino acids produced by the hydrolysis of a protein. Within certain concentration limits the amount of leucine was found to be approximately proportional to the dry weight of leucineless cultures produced.

The mold *Neurospora crassa* and its mutants not only are aids in bioassay in determination of various growth factors, but are also increasingly important tools in the determination of the sequence of events in chain reactions produced by the cell. The original or "wild" strain of *Neurospora* will grow on a simple chemically defined medium, as for example on a solution of sucrose, nitrate, biotin, and certain inorganic salts. From these it produces a great variety of compounds. Beadle and Tatum and associates have shown that it is possible to induce mutations which will not grow in the simple synthetic medium but will grow in one which is complex. It is then possible to determine which compound present in the complex mixture will when added to the simple mixture enable the organism to grow. By use of various mutants it has been possible to determine compounds produced in a series of reactions in which each compound in the chain is produced from the preceding by a specific enzyme. For example, a mutant was found to require tryptophan. The ability to synthesize tryptophan had been lost. Two other mutants were found, one required the presence either of indole or tryptophan, and the other required one of the three compounds, anthranilic acid, indole, or tryptophan. Let A, B, C, D, E be a series of compounds each derived from the preceding.

$$A \xrightarrow{1} B \xrightarrow{2} C \xrightarrow{3} D \xrightarrow{4} E$$

Each change, 1, 2, 3, 4, is produced by a specific enzyme. If E is essential to the growth of the organism, growth will stop if any one of the four enzymes is absent. If enzyme 4 is lacking, the organism must have E. If 3 is absent and 4 present, then normal growth will occur in the presence of either D or E. If 1 is absent, the mold can use B, C, D, or E. The importance of this tool in getting at the cell growth mechanisms is apparent.

One other consequence of the blocking of one step in a chain reaction of the type just illustrated should be noted. If reaction No. 2 is blocked the compound B continues to be produced from A by reaction 1, and B will accumulate in the medium. It has been shown in some cases that such accumulation may interfere with the essential reactions and prevent normal growth. Work thus far accomplished seems to show that single genes in the cell control single biochemical reactions, apparently by controlling the production of specific enzymes.

This technique of the use of organisms for determining steps in a sequence of chemical changes may be used not only for study of synthesis but for study of stages in the breakdown of complex compounds. For example, Lederberg (1948) has shown that normal utilization of the sugar lactose by *Escherichia coli* may be affected by mutations of at least seven quite independent genes.

Bonnet (1948) has indulged in some interesting speculations as to the number of enzymes apparently required in the metabolism of an organism. Geneticists have suggested that the cell may contain genes to the number of 10,000 more or less. If there is a one to one relationship between gene and enzyme, would the 10,000 possible enzymes be enough to explain the chemical changes required by a living cell? He concludes that such a number is probably sufficient.

Many kinds of bacteria have likewise been found useful in determination of "growth factors." In some cases the organism used is one which, when first isolated was dependent upon the presence in the medium of certain compounds. For example, there are several species of pathogenic bacteria that were first cultivated satisfactorily when certain body fluids as blood serum were added to the medium. The bacterial genus *Hemophilus* gained its name because the various species apparently required blood. Tomato juice accelerates the growth of many organisms. Many and careful studies were made to determine what special constituents of such materials as tomato juice were stimulating or essential. Gradually a long list of such essentials for particular organisms has been compiled. In other cases treatments to induce mutations have been successful, and strains have been developed having special growth requirements. Among the bacteria found useful are *Streptococcus fecalis* for the estimation of pyridoxine and nicotinic acid, *Clostridium acetobutylicum* for assay for para-amino benzoic acid (abbreviated as PAB), a strain of *Shigella paradysenteriae* for uracil, a strain (9790) of *Streptococcus fecalis* for arginine, threonine, tryptophan, and valine, *Leuconostoc mesenteroides* for histidine, lysine, methionine, and phenylalanine, *Lactobacillus arabinosus* for isoleucine and leucine.

Where large numbers of determinations (bioassays) are to be made, Nyman, Gunsaulus, and Gortner (1945) found the task of maintaining suitable cultures of required bacteria greatly simplified by use of the technique of lyophilization. For example, cultures of *Lactobacillus arabinosus* useful in certain bioassays could be prepared in sufficient quantities to last for several months to a year. They found that uniform inocula could be produced by centrifuging broth cultures, adding a small amount of milk to the sediment, distributing in one-tenth-milliliter lots in small tubes, freezing, drying in a vacuum, and sealing each tube to maintain the vacuum. This procedure insures uniformity in inoculation.

Protozoa are also being used with marked success for bioassay and for studies of cell metabolism. Particularly striking has been the work of Kidder and associates who have found that protozoa of the genus *Tetrahymena* have many of the same nutrient requirements as man, and diet adequacies can be quickly determined. This organism can be grown much as are the bacteria in a peptone medium. It was known that at least three groups of factors were required for growth. The complexity of the situation may be indicated by the finding that the factor II, not precipitable by lead acetate, could itself be resolved into two distinct factors termed IIA (named *protogen* by Stokstad and associates) and IIB. The latter was again fractionated. A complete synthetic medium giving good growth of *Tetrahymena* has been developed. It was found that of the amino acids eleven were either essential or their absence greatly restricted growth, and in addition eight other amino acids were somewhat stimulating but not essential. Organic growth factors found to be essential numbered seven, and in addition three others were stimulating. As energy sources, growth was stimulated by glucose and sodium acetate. Salts of magnesium, potassium, calcium, iron, and copper, with the anions sulfate and phosphate were essential, with traces of manganese and zinc stimulatory.

CHAPTER 23

Microorganisms in the Production of Antibiotics and Similar Compounds

Several compounds resulting from cell syntheses have recently come into prominence because of their use in the treatment of disease. These are substances which are produced most commonly by microorganisms, at least by living cells, and which are inimical to the growth of pathogens.

Symbiosis is the living together of organisms which are mutually beneficial. The term includes not only the relationship which sometimes exists between microorganisms, but also between microorganisms and higher plants and animals. This type of mutual aid or *symbiotic activity* is so common and so important from an economic point of view that it will be considered in a separate chapter (Chapter 27).

Synergism or *synergistic action* as applied to microorganisms means that two kinds growing together may be able to bring about changes, such as fermentations, which neither could produce alone. In the discussion of chemical changes induced by organisms it was noted that some of the same basic mechanisms may be important in many of these fermentative changes. For example, one bacterium is able to produce acid but no gas from the disaccharide sucrose, another is able to produce gas from the monosaccharide glucose but not from sucrose. When the two organisms are grown together in the presence of sucrose, both acid and gas are produced. In some cases synergistic action results in a marked increase in activity in one organism due to the presence of another. This may be due to the production by the second organism of some growth accelerant. This, for example, seems to be the explanation of the *satellite* phenomenon observed in plate cultures of bacteria in which colonies of one organism are markedly increased in size and vigor when they develop in close proximity to colonies of certain other species.

Another term used to designate the favorable effect of one organism upon the growth of another is *metabiosis*. One organism, by its growth, paves the way for another which may utilize its growth products. For example, yeast ferments sugars with production of alcohol under essentially anaerobic conditions. Bacteria of the genus *Acetobacter* under aerobic conditions utilize the alcohol and transform it into other compounds, particularly acetic acid.

The phenomenon of *commensalism* intergrades with metabiosis. One organism is harbored by, or its growth is favored by, another without there appearing to be the mutual benefit characteristic of symbiosis on the one hand nor on the other the deleterious effect on one, characteristic of parasitism. The bacterial and protozoan flora and fauna of the alimentary tract of man and animals are usually quoted as examples of commensalism, the organisms not injuring the host. However, some of these cases are being found to have some of the characteristics of symbiosis; the organisms of the alimentary tract may perform very important digestive functions and synthesize and provide essential vitamins for the host.

Antibiosis is the manifestation of a deleterious effect by one organism upon another without true parasitism, usually as the result of the production by one organism of a substance or substances which will inhibit the growth of another organism or even kill it. The effect may be *bacteriostatic*, in which case the other organism is prevented from growing, or it may even be *bactericidal* or *fungicidal* in action. These substances, termed *antibiotics*, constitute a special class of inhibitors or germicides. The substances produced may be inhibitory also to the organism forming them. Such self-inhibition is sometimes termed *autoantibiosis* and the compounds produced, *autotoxins* or *staling substances*. The latter name is given because the medium which has supported growth of the organism will no longer permit its growth —it has become *stale*. It may be shown in suitable cases that the staling is due to the presence of inhibiting substances and not to exhaustion of nutrients in the medium.

Antibiotics and Their Development. The great quest of the Middle Ages was for reliable cures or specifics for disease. As a result of long study and much empiricism certain drugs were discovered which were useful. Some were inorganic in nature, as mercury, others were of vegetable origin. Great volumes on materia medica and bulky pharmacopoeias were developed. With the establishment of the germ theory of disease, search for specifics was greatly intensified. Some were found to be produced by higher plants, as the alkaloid quinine from the bark of the cinchona tree, so useful in combating malaria. Some were developed through chemical synthesis by careful survey of all compounds related chemically to those which proved useful. Other specifics were found in the antibodies present in the blood serum of persons or animals immunized against a disease, i.e., such substances as diphtheria antitoxin. Still more recently it has been found that certain kinds of microorganisms, particularly certain fungi and bacteria, produce substances which antagonize other microorganisms; in other words, it was found that not only do higher plants produce substances which may be used medicinally, but that they are formed by certain molds and other microorganisms as well. Somewhat inappropriately, the term *antibiotic* (*anti,* against; *bios,* life) has been restricted in practical use to substances produced by microorganisms found antagonistic to other organisms. The number of these and their relative importance warrants a review of their origin and characteristics.

That certain organisms produce antibiotics has long been known. In routine plating of materials, such as soil, in the laboratory, it is frequently observed that in thickly seeded plates certain colonies are surrounded by a clear zone in which no other colonies develop. Evidently the central colony produces some substance, some "antibiotic," which diffuses into the medium and stops colony formation on the part of other bacteria present. The formation of lactic acid in the fermentation of certain foodstuffs, as cabbage in the production of sauerkraut, effectively prevents the growth of undesirable bacteria and eventually so increases the hydrogen ion concentration as to stop the growth of the lactic bacteria themselves. This type of microbial antagonism has long been known, and the term antibiotic is not usually applied to substances which inhibit by changes in pH, oxidation-reduction potential, or changes in surface tension.

The fact that one organism may produce substances deleterious to others early led to therapeutic tests. The substances first studied were not found to be of particular benefit in medicine; only within recent years there have been found those that are of great service in therapy.

Pyocyanase. Apparently the first antibiotic studied and named was *pyocyanase*. The organism responsible for its production is a member of the genus *Pseudomonas*. The organism was first described by Schroeter

(1872) and named *Bacterium aeruginosum*[1] because of the greenish blue pigment formed in a suitable medium. Later, the organism was rediscovered by Gessard in 1882 and termed *Bacillus pyocyaneus*. It was studied under this name by Bouchard (1888), who found that a product of its growth would inhibit the growth of *Bacillus anthracis*, the cause of anthrax, and of many other bacteria. This inhibiting substance was studied by numerous workers. By filtration to remove the bacteria, evaporation, dialysis, and precipitation, a preparation called *pyocyanase* was secured by Emmerich and Low. The material is soluble in alcohol and in ether and is resistant to boiling temperatures. It is probable that the effect upon other bacteria is due to the unsaturated fatty acids present. Pyocyanase tends to dissolve many bacteria, both gram-positive and gram-negative forms. The pigment pyocyanin produced by this organism also has antibiotic activity. Various other preparations have been extensively studied, but no satisfactory therapeutic use of *Pseudomonas aeruginosa* and its growth products has been developed.

Other Antibiotics from True Bacteria. There are numerous accounts in the literature relative to the antibiotic activity of many kinds of bacteria. Antibiotics have been isolated, characterized, and named in some cases. *Gramicidin* is produced by *Bacillus brevis*, a spore-bearing rod not uncommon in soil. Gramicidin may be crystallized and is soluble in alcohol, ether, and acetone. It destroys gram-positive bacteria. *Subtilin* is produced by *Bacillus subtilis* and also destroys gram-positive bacteria. *Tyrocidine*, also produced by *Bacillus brevis*, is of some special interest because in composition it is a polypeptide with a molecular weight of about 1260 and among its products of hydrolysis are at least eight α-amino acids.

None of the antibiotics produced by true bacteria have thus far found extensive use in medicine, although active work is continuing in this field.

Antibiotics from Molds. Dr. Alexander Fleming (1929) isolated a strain of a species of *Penicillium* (*P. notatum*) which he showed to be highly antagonistic to many bacteria and to produce an antibiotic which was named *penicillin*. It was found to be relatively non-toxic when injected into animals and to destroy the bacteria of many diseases, or perhaps better, it enabled the body to suppress and eliminate the bacteria. The results were on the whole spectacular. Response to treatment with penicillin in certain diseases was prompt and satisfactory. This antibiotic has become one of the most widely known and used of therapeutic agents. In the United States alone, commercial production amounts to hundreds of billions of units annually.

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[1] The Latin adjective *aeruginosum* means full of copper rust or verdigris, the green of oxidized copper.

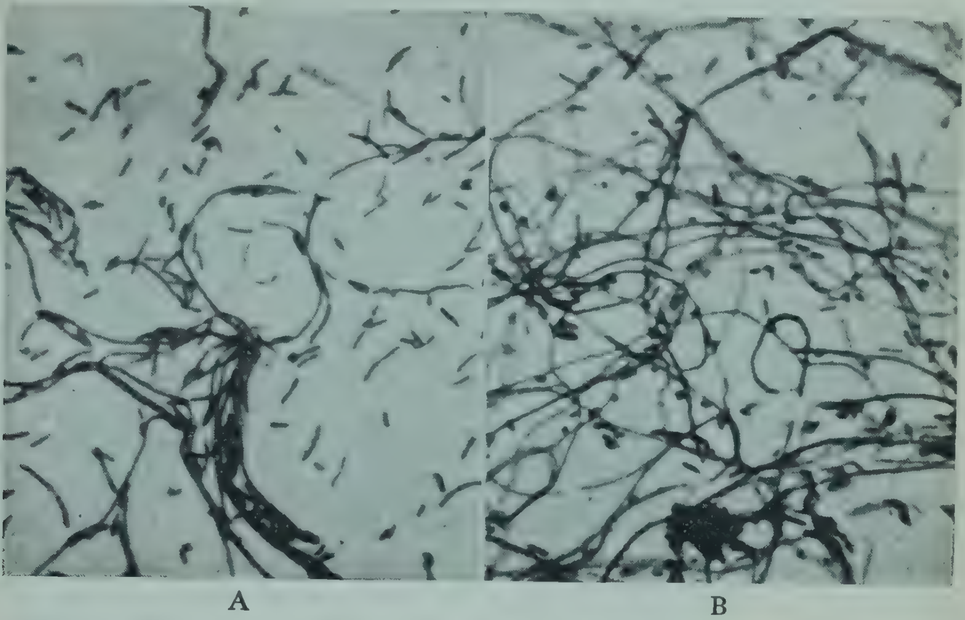


Fig. 23–1. Effect of penicillin on bacterial morphology. A. On *Salmonella enteritidis*. Note filaments. B. On *Salmonella oranienberg*. (Courtesy Dr. Max Levine.)

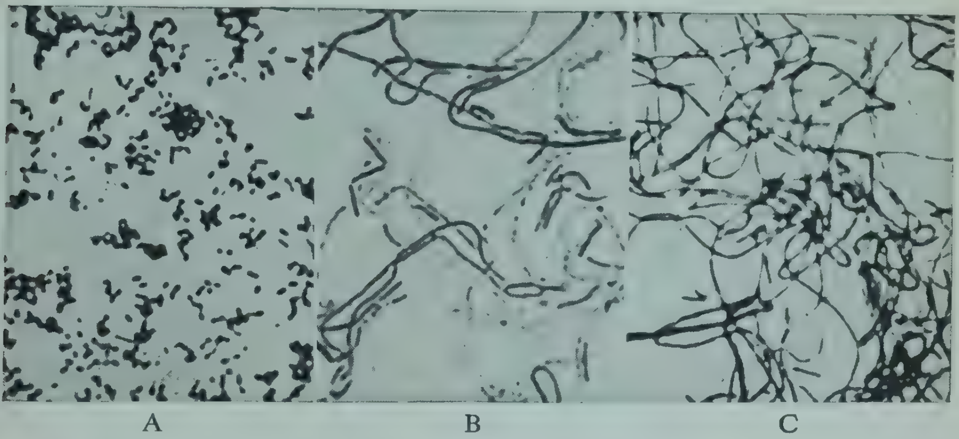


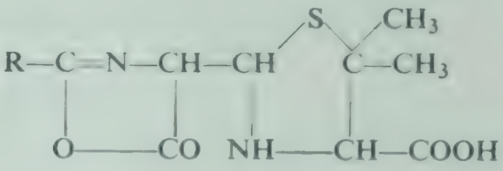
Fig. 23–2. Effect of penicillin upon growth of *Proteus*. A. Normal cells. B. Filamentous and ghost cells. C. Filamentous cells. (Courtesy Dr. Max Levine.)

Several species of penicillia are known to produce penicillin or similar compounds. The species first and still generally used is *Penicil-*

lium notatum. It resembles and is closely allied to the blue-green molds frequently seen on moldy oranges and other foods. *Penicillium* is essentially aerobic in its growth. It was first cultivated on the surface of liquid media in relatively shallow layers. Later production was expedited by growing the organism submerged in a liquid medium which is kept thoroughly aerated. The active penicillin produced is extracted from the medium by suitable solvents and concentrated.

Many variations in composition of medium have been tested in an endeavor to get maximum production. The solution usually used contains lactose, glucose, acetic acid, salts, and a suitable source of nitrogen, as ammonium nitrate. In addition certain growth factors present in corn steep liquor or cotton seed meal are added.

Much study has been devoted to determining the chemical structure of penicillin. It was soon discovered that there were several related antibiotics (at least six) produced by different strains or species of *Penicillium* under different growth conditions. They have been found to differ not in their basic structure but in the character of side chains. The basic structure may be represented as



The R of this basic formula may be any one of six radicals. For example, the side chain R of penicillin F is $CH_3{\cdot}CH_2{\cdot}CH{:}CH{\cdot}CH_2$—.

The development of better media, of better knowledge of favorable environment, and the selection of more efficient strains of *Penicillium*, and particularly the development of mutant strains through exposure to x rays and to ultraviolet light have led gradually to an increase in the production of penicillin to as many as 1000 or even 2000 units per milliliter.

The penicillins are relatively strong acids from which the sodium or calcium salts are usually prepared for therapeutic use.

Standardization of penicillin was developed by Florey who proposed the use of what has come to be known as the Oxford unit. The unit of penicillin is that amount which when mixed with fifty milliliters of a standard meat extract broth will inhibit the growth of a standard strain of *Micrococcus aureus*. Later, by international agreement through the World Health Organization of the United Nations, a certain lot of pure crystalline penicillin G was adopted as the standard, and the unit was fixed as 0.6 microgram of this standard, an amount equivalent to one Oxford unit.

Several methods of determining concentration of penicillin have been proposed. That most often used is to seed nutrient agar plate heavily with the test organism (*Micrococcus aureus*), place on this agar several (frequently six) short glass or porcelain tubes, forming shallow cups. In one of these a solution containing one unit of penicillin is placed, in the others various concentrations of the material under test. The penicillin from each cup will diffuse through the agar and prevent the growth of the bacteria. After incubation each cup will be surrounded by a zone of no growth. The zone will be wider the greater the concentration of penicillin originally placed in the cup. That cup which produces a zone of inhibition equal in diameter to

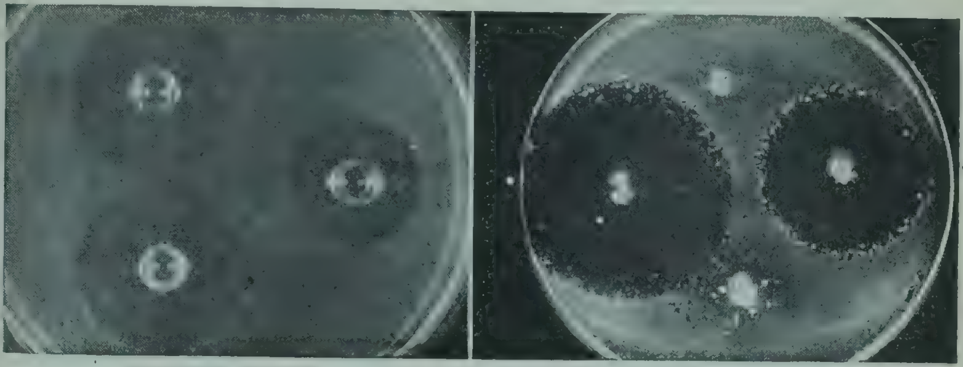

A B

Fig. 23–3. A. Cup method of testing the concentration of an antibiotic. An agar plate is heavily seeded with *Bacillus subtilis*. Three glass cylinders, sterile, have been placed on the surface, and a dilute solution of streptomycin placed in each. In the cylinder to the right have been placed 15 units of streptomycin in one milliliter of water. Note that the *Bacillus subtilis* has been inhibited from growing in a zone. In each of the other cylinders there have been placed 60 units of streptomycin. Note that the zones of inhibition are wider. In a series of such tests, any cylinder which gave a zone of inhibition of the same width as the one to the right would be recognized as containing fifteen units of streptomycin. Standardization of preparations of streptomycin are possible through this technique. (Courtesy of Dr. Selman A. Waksman.) B. Antibiosis of cultures of bacteria. The agar plate has been thickly seeded with the test organism, then inoculated at four points each with a bacterial culture. Note that there is little evidence of inhibition of the colonies of the test organism about the upper colony, some inhibition about the lower colony, marked inhibition about the other two colonies. Observe also that in many cases the colonies on the margin of the inhibition zone seem to be stimulated and that there are occasional colonies that have developed within the zone of inhibition. (Courtesy of Dr. Frederiq.)

that produced around a cup containing the known standard unit must itself contain one unit.

Some organisms, particularly bacteria, are known to produce a specific enzyme *penicillinase* which rapidly destroys penicillin. It is therefore possible to secure races or strains of organisms that are not affected by penicillin. For example, penicillin is usually highly effective in the destruction, both in the test tube and in the body, of the pathogenic *Micrococcus aureus* or staphylococcus. However, strains are sometimes found which are resistant due to production of penicillinase.

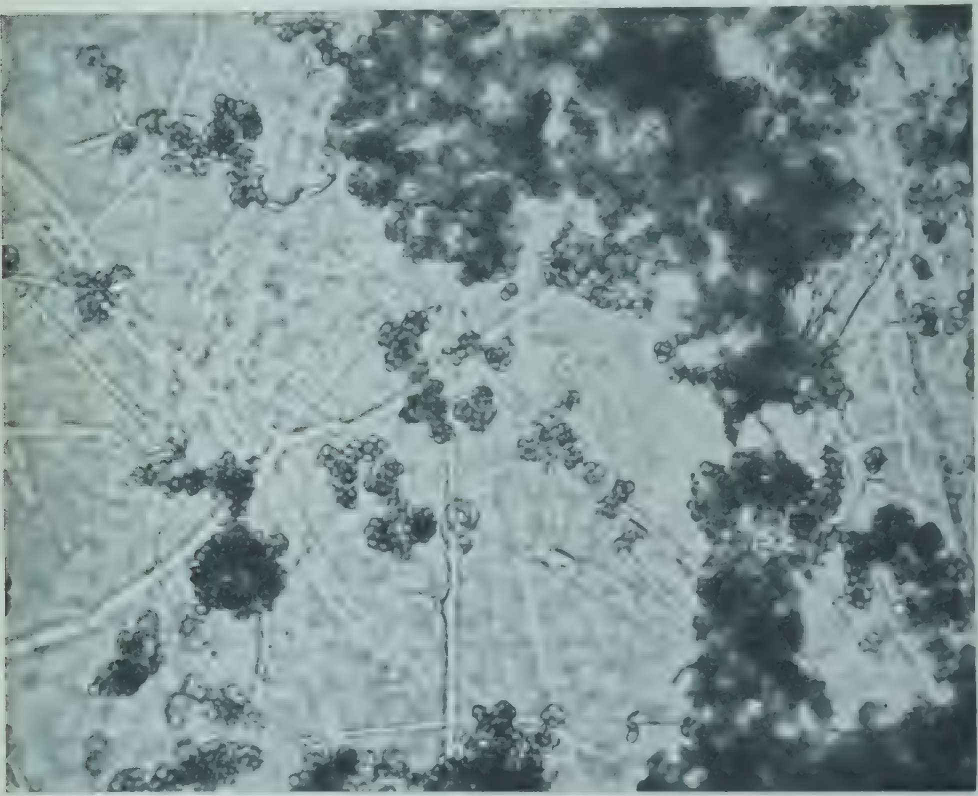


Fig. 23–4. *Trichoderma,* a mold which produces gliotoxin. Mycelium, and dark single-celled spores.

Among other antibiotics produced by molds are the following.

Citrinin is the characteristic pigment isolated from cultures of *Penicillium citrinum.* This organism produces a yellow pigment which diffuses into the medium. It may be isolated from the clear broth filtrates by acidifying with normal hydrochloric acid to a pH between 2.5 and 3.0. Yellow crystals of the citrinin precipitate and may be removed by filtration. This antibiotic is one of the easiest to prepare, large yields may be secured, and the compound is quite stable. It is apparently too toxic when injected into animals to be safe for use internally, but it has been claimed to be efficient in local treatment of wounds or surface infections.

Clavacin, patulin, claviformin are probably identical or closely related antibiotics produced by the fungi *Aspergillus fumigatus, Penicillium patulum, P. claviforme,* and *P. expansum.* They are somewhat toxic to animals, but markedly effective against gram-negative bacteria.

Gliotoxin is formed by various species of the mold genera *Gliocladium* and *Trichoderma.* It stops the growth of certain fungi which attack plant roots, such as *Rhizoctonia.* It also will inhibit or kill bacteria. It is so highly toxic to animals as to be of little therapeutic use.

Antibiotics Produced by Bacterial Species of the Order Actinomycetales. Waksman and his associates, and later other investigators, have made noteworthy contributions to our knowledge of antibiotics produced by various actinomycetes. Waksman and Woodruff (1940) named two substances produced by *Streptomyces antibioticus, actinomycin A* and *actinomycin B.* The former is a bright red pigment having high powers of bacteriostasis, but weakly bactericidal. The actinomycin B is colorless and strongly bactericidal. These proved toxic to animals. *Streptothricin* (1943) is a product of the growth of *Streptomyces lavendulae,* when cultivated on a suitable medium, preferably containing starch. This compound was found to be relatively low in toxicity to animals and highly germicidal for gram-negative bacteria. These antibiotics appear to be of historical interest only.

A more important antibiotic *streptomycin* was isolated by Waksman (1944) and associates from *Streptomyces griseus,* an organism which had previously (1925) been isolated and named by him. Streptomycin is highly bactericidal toward many gram-negative organisms and to some gram-positive forms. It is relatively non-toxic and has proved to be particularly useful in combating certain types of tuberculosis. It has come to be an important therapeutic agent, being active in some cases where the penicillin is relatively ineffective. Streptomycin has been determined to be an unusually complex compound, consisting of two principal components, streptidine and streptobiosamine, both of them also relatively complex. The organism is grown in a slightly modified glucose broth. Streptomycin is produced commercially in large quantities by the use of aerated submerged cultures.

The group of the actinomycetes has proved to be very productive of antibiotic substances; many have been produced and named, some of them apparently of considerable value. One of the most interesting of these is *aureomycin* produced by *Streptomyces aureofaciens*. This antibiotic has been used with a considerable degree of success in the treatment of a variety of diseases, including some produced by viruses that had proved resistant to treatment with other antibiotics.

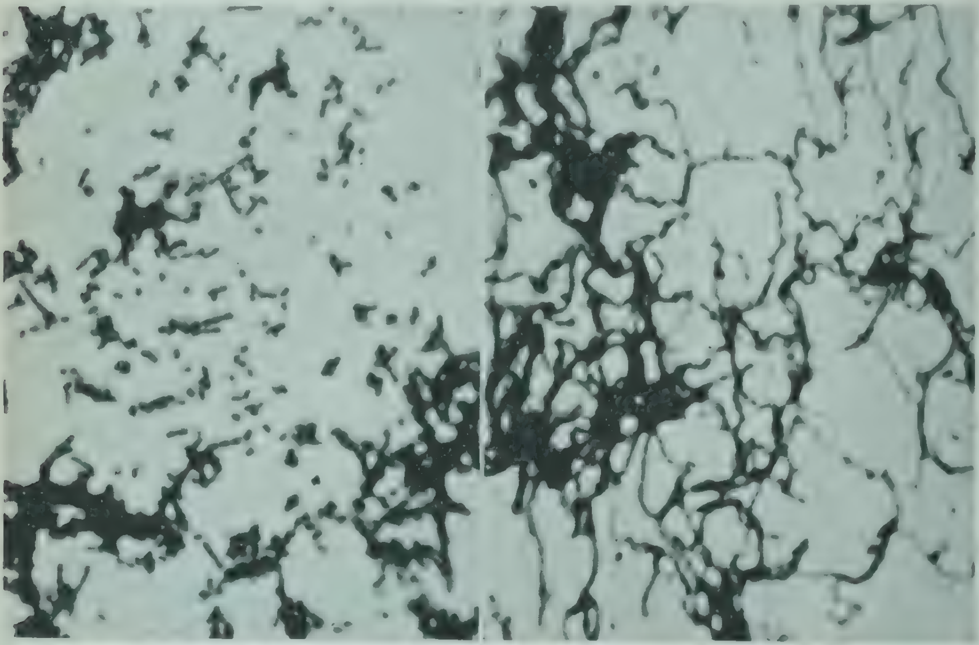

A B

Fig. 23–5. *Streptomyces griseus*, the organism which produces the antibiotic streptomycin. A. The chains of spores produced from the mycelium. B. The branched mycelium.

Of special significance has been the finding that this organism, and probably some others, also produce in the course of their growth considerable amounts of the so-called *animal protein factor* (APF), probably B_{12}, which when fed to omnivorous animals such as swine enables them to make much more efficient use of vegetable proteins, in fact in part eliminating the necessity of including animal proteins in the diet as a requirement for optimum growth. This finding has led to the production and utilization of the byproducts of the maufacture of aureomycin in the preparation of commercial feeds for swine and for poultry. It is also claimed that the presence of traces of aureomycin in these byproducts has a favorable influence in minimizing occurrence of certain types of enteritis in swine, and in materially speeding up gain in weight.

Still other antibiotics produced by species of *Streptomyces* which have proved useful are *chloromycetin* and *neomycin*.

Mode of Action of Antibiotics. Antibiotics are not uniform in their effect upon microorganisms which they inhibit. Studies on various types have shown that in some cases, as with penicillin, the antibiotic interferes with the passage of amino acids through the cell membranes. Apparently streptomycin inhibits the activity of certain enzymes in the so-called Krebs cycle; probably tyrocidin changes the permeability of the cell membranes so that some of the cell contents may diffuse to the outside and be lost.

An explanation of the activity of certain of the antibiotics that are peptides in structure, as gramicidin, may be found in the fact that, in part, the amino acids constituting the compound are of the *d*-configuration instead of the *l*- which is the type usually found in cells and the type used in cell metabolism. Apparently, the *d*-amino acid unites with the enzyme which catalyzes the reactions to be produced in normal metabolism and blocks the catalysis of the *l*-acids present. For example, it was shown by Fox (1944) and associates that growth of *Lactobacillus arabinosus* is inhibited by the unnatural *d*-leucine even in the presence of concentrations of the normal *l*-leucine two hundred times as great. Others have found similar effects of *d*-serine on the growth of *Escherichia coli* and on toxin production in cultures of *Clostridium tetani*. It is evident that to be useful in combating disease organisms in the animal body the action of some enzymes of the organism would need to be blocked without the compound interfering with the normal metabolism of the cells of the host animal.

A very interesting and important corollary would be to locate some enzymatic activity normal to tumor or cancer cells but not essential to the growth of the normal cells of the body and endeavor to develop compounds which would block such enzyme action. Kidder (1949) and associates have definitely pointed this out as a hopeful lead in the inhibition of growth of tumor cells. Work with the protozoan *Tetrahymena geleii* showed that it had very much the same requirements for nutrients for growth as do higher animals, but with certain exceptions. The protozoan requires guanine and uracil, while mammals in general can synthesize them from related compounds, and do not utilize them in their diets. These workers found that a compound, *guanazolo* (an abbreviated designation of a complex compound having, in part, the configuration of guanine), if added to a completely synthetic medium capable of supporting growth of the protozoan, would inhibit growth. Such a compound might be termed a wholly synthetic antibiotic. But most interesting was the finding that development of certain tumor cells in the mouse apparently is inhibited by the same compound, without apparent injury to the normal tissues. The importance of this kind of a lead in cancer research is obvious.

CHAPTER 24

Microorganisms in the Preservation and Processing of Foods and Feeds

Bacteria, yeasts, and molds, occasionally even viruses such as the bacteriophages, play an important role in the processing and the preservation of many substances used as food for man, feed for animals, fibers for clothing and other purposes, and of such products as leather and wood. In some cases we are interested in these organisms because they bring about unwanted changes and cause spoilage, and we become primarily concerned with preservation and the development of methods that may be used to prevent deterioration. In other cases the changes produced by the organisms are classed as desirable and useful in *processing*. For example, the acids developed by organisms in the processing of foods contribute to the flavor of the final product and also act as preservatives, preventing the growth of undesirable organisms. It is desirable to discuss first the objectionable organisms and how their growth may be prevented, that is, *preservation,* and then to discuss the participation of microorganisms in *processing* of foods, feeds, fibers, and other materials.

PRESERVATION AGAINST DELETERIOUS CHANGES PRODUCED BY MICROORGANISMS

It is necessary that foods and feeds be protected from the destructive action of microorganisms for considerable periods of time in order that the abundance of the harvest season may be conserved to supply the needs of the entire year. Nature herself preserves many foods by drying and by protective coverings of various kinds. Man has added to the methods of preservation until it is now possible to keep practically any kind of food from the period of its greatest abundance through the period of its greatest scarcity. In some cases the need for preservation is limited to a few hours or days, in other cases foods, feeds, and other products subject to injury by microorganisms must be stored for months or even years.

Agencies Destructive to Food Materials

Before discussing methods of preservation against injury or destruction by microorganisms, those agencies which tend to bring about undesirable changes should be identified. They may be grouped under two general headings: first, the *intrinsic,* including those which are normally present in the material itself and have not been derived from outside sources; second, the *extrinsic,* those which enter at some time during the period of preparation, ripening, or processing.

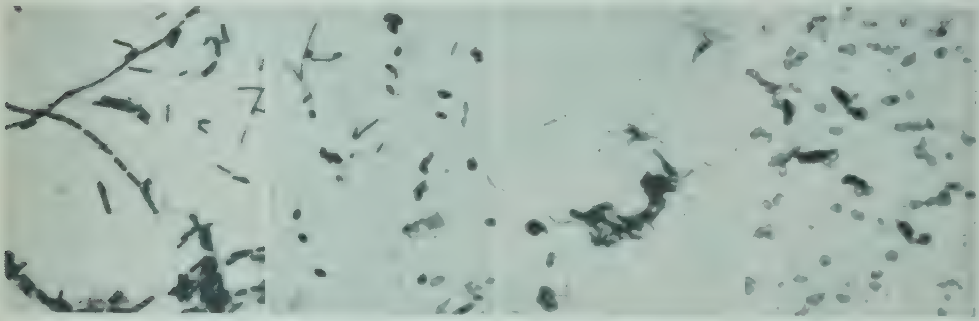


Fig. 24–1. Bacteria from decaying food, showing different types and mixtures found in such situations.

To the *intrinsic agencies* belong the so-called *autolytic enzymes.* In certain fatty foods, for example, there may be normally present hydrolyzing enzymes which will bring about rancidity. In fruits and certain vegetables the enzymes which normally bring about ripening may eventually produce a condition of over-ripeness. Certain of the oxidizing enzymes, such as those present in fruits, may produce discoloration.

The *extrinsic agencies* capable of bringing about deterioration are the *bacteria,* the *yeasts,* and the *molds,* together with the *enzymes which they produce* and occasionally the viruses or *phages* which parasitize them. Which is most important will depend upon the character of the food and the conditions under which it is preserved. In general the *bacteria* thrive best in nitrogenous foods containing a considerable proportion of water and not too much acid; they grow best in a medium which is nearly neutral. *Yeasts* for the most part grow best in a medium which is rich in carbohydrates, particularly in the fermentable sugars, and preferably in the presence of some acid. Like the bacteria, they prefer a considerable amount of moisture. The *molds* will grow in media somewhat drier and of almost any type. Certain molds will grow on foods that are highly acid as well as upon those which are neutral or slightly alkaline.

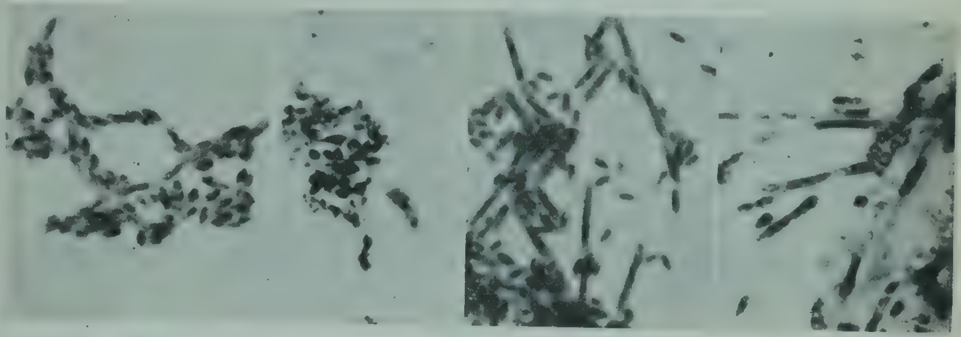


Fig. 24–2. Yeasts, non-filamentous and filamentous as found in various fermentations of food.

Methods of Food Preservation

Food is most commonly preserved, that is protected from attack by organisms, by one of four methods: by the use of *high temperatures* or *heat,* by the use of *low temperatures* or *cold,* by the use of *preservative substances* of various kinds, and by *drying.*

Preservation of Food by Heat. All types of food not damaged or seriously changed by the application of heat may be preserved by this means. Among the foods so preserved are fruits, vegetables, meats, and fish. The process in all cases consists in the application of a sufficient degree of heat to destroy all organisms, or all of certain types of organisms, and thereafter in maintaining the food under conditions such that organisms cannot gain admission or, if present, cannot develop.

Two principal methods of heat application are used, pasteurization or sterilization. In *pasteurization,* the food is raised to such a temperature that the organisms of certain types, but not necessarily all organisms, are destroyed. In some cases, this results merely in a prolongation of the time during which food may be held before use, in other cases it permanently inhibits certain undesirable fermentations. *Sterilization* by heat implies the use of a temperature such that all organisms are destroyed, or at least all those that are able to grow in the food under the conditions under which it is kept.

Pasteurization. This process is ordinarily applied to milk and cream and to certain alcoholic beverages, particularly beer and wine. Milk and cream are pasteurized for several distinct purposes. (1) They may be heated to destroy any disease-producing bacteria that may be present. (2) The pasteurization of milk destroys many, though usually not all, of the lactic acid bacteria present and greatly retards the production of lactic acid and the souring of the milk. (3) Pasteurization of cream is carried out by creameries in an effort to destroy most of the bacteria present, after which it is inoculated with certain desirable lactic acid bacteria or "starters." Beer and sometimes wine are pasteurized to destroy the yeasts present and thus stop fermentation, also to kill organisms which might give rise to undesirable flavors or aromas. Inasmuch as pasteurization is most commonly applied to milk, consideration of this topic will be deferred to that section of this chapter in which milk deterioration is discussed.

Sterilization. Food materials are heated to such a temperature and for such a time as will destroy all the living microorganisms present and so sealed that microorganisms cannot enter may be preserved indefinitely. The process most commonly used for this purpose is that in which the food material either before or after sterilization is hermetically sealed in containers. Meat, fruits, vegetables, and in general those foods not seriously damaged by heat and which cannot be satisfactorily preserved by drying are utilized.

In any method of canning it is necessary that the food be *properly prepared*, placed in *suitable containers*, the *air exhausted*, and the food *hermetically sealed*, and *sterilized*. We are here concerned primarily with the factors which determine the time and the temperature necessary for sterilization. Some six of these are worthy of emphasis.

1. INITIAL CONTAMINATION. The first of the factors determining the time necessary for sterilization of the food is the extent and kind of the initial contamination. All the microorganisms initially present are not killed off instantly by heat, rather the temperature determines the *rate of death*. In general, the *larger the initial number of microorganisms to be destroyed the longer will be the time necessary to kill all.* The kinds of organisms present will also influence the time needed. Spore-producing bacteria are more difficult to destroy than the nonsporing types. Washing or use of other means to diminish the number of bacteria will influence the ease of sterilization.

Blanching affects the numbers of bacteria present and is used as a preliminary step in the canning of many foods. The products, usually vegetables or fruits, are dipped first into hot water, then into cold water. This process shrinks the tissues somewhat, makes them relatively firmer, tends to set the color, and at the same time removes many of the bacteria which were present, thus reducing the initial infection and presumably decreasing the length of time necessary for sterilization. It has been claimed by some writers that blanching also renders the bacteria which remain more readily destroyed when first heated, then cooled (or chilled), and reheated. Several tests, however, have failed to show any marked effect of this "cold shock"; blanching apparently does not make bacteria more susceptible to higher temperatures.

2. SIZE AND SHAPE OF CONTAINER. The center of any container usually is the last part to reach the desired temperature; therefore, the larger the container the longer will it take. The shape of the container will also determine in part the distance which heat will have to travel to the center.

3. HEAT CONDUCTION AND CONVECTION IN THE FOOD. The ease with which heat may penetrate the food is important. Where the particles are relatively firm and are surrounded by water or thin syrup, convection currents are at once set up in the liquid when heat is applied to the can and the interior heats relatively quickly by a process analogous to that used in a hot-water system in the heating of a home. If the material surrounding the food particles is viscous or gelatinous, or if there is not much (if any) free water, convection takes place slowly or not at all. Heat passes to the center through the contents much more slowly and only by *conduction*. Cans of corn heat more slowly at the center than do cans of cherries; substances like pumpkin and spinach heat most slowly of all. When there are no convection currents, the rate of heat penetration through food is practically the same as the rate of heat conduction through water.

4. ACIDITY, The hydrogen ion activity (pH), the true acidity of the food material, is also important in fixing time required for sterilization. Foods such as peaches or apples are much more easily sterilized by heat than are vegetables such as peas, beans, and corn. The presence of acid greatly increases the death rates of microorganisms at high temperatures. In some cases acids such as vinegar or lemon juice are added; such amendments decrease materially the time and the temperature needed for sterilization.

5. AGITATION. In commercial canneries devices (agitators) are occasionally employed to keep the canned foods continually in motion during sterilization. This mixes the contents and increases markedly the rate at which the heating will occur. Agitation greatly increases

convection, making it significant even in viscous or gelatinous foods.

6. TEMPERATURE. The time required for sterilization is also influenced by the temperature to which the food is subjected.

Three methods of food preservation by heat are in common use in which the temperature is that which may be secured at atmospheric pressure. In the *open kettle* method the material to be preserved is heated in an open vessel, then poured into the hot, sterile containers (as Mason fruit jars), and sealed. The highest temperature reached is the boiling point of the material to be preserved. When this contains considerable quantities of sugar or other solutes the boiling point may be several degrees above the boiling point of water. This method is most effective with acid fruits and vegetables and with pickled vegetables and fruits.

In the *cold pack* process the foods are placed in jars with covers adjusted and heated in boiling water or in streaming steam. The temperature of the contents of the can will consequently never rise higher than 100° C. The character of the material canned and the other factors here listed determine the length of time which must be used in order to secure sterility. With acid fruits a short time only is necessary; with vegetables such as corn and beans several hours at least are required, and results are somewhat uncertain.

Intermittent sterilization has been extensively used in some parts of the United States. The cans containing the material to be preserved are placed in water, the water is brought to the boiling point and kept at this temperature for a period of an hour or more. The cans are then allowed to cool, and the process is repeated on three or more successive days. This is the principle of intermittent sterilization such as is used in the laboratory for sugar media easily destroyed by heat. The underlying theory assumes that the spores present begin to germinate in the first twenty-four hours. The second heating will kill all the vegetative forms which have developed. It has been contended that repeating the heating in this manner several times will quite certainly destroy all the bacteria which may be present. In general this process is not advocated, although when properly carried out it usually is successful. The most serious danger is possible failure to destroy the spores of the organism producing botulism, a type of food poisoning.

The *pressure cooker* in the home and the large processing retorts in the commercial canneries have made possible the use of steam under pressure for heating and the consequent securing of temperatures much higher than the boiling point of water. Experience has developed, for each type of canned food, the time and pressure necessary for adequate sterilization. Unquestionably sterilization in the pressure cooker, when properly carried out, is safer and will lead to better results in most cases than other methods, particularly in the canning of non-acid vegetables and meats.

Not all canned foods, even though they may keep perfectly well, are completely sterilized. It has been determined as the result of many studies that the microorganisms which may bring about deterioration in canned foods are: (1) those which may enter the food as the result of defective sealing; (2) those aerobic spore-producing bacteria which normally are unable to grow in canned foods because of the complete exclusion of air but which may find conditions suitable for growth if air is admitted due to defective sealing; (3) anaerobic spore-producing bacteria capable of growing at normal temperatures, which occasionally escape sterilization and produce deleterious changes, frequently accompanied by evolution of gas and the development of malodorous and bad-tasting compounds. Some of these microorganisms may produce poisons or toxins (such as *Clostridium botulinum*); (4) certain of the most resistant of the spore-bearing bacteria are thermophiles. Their unusually resistant spores will seldom germinate unless the canned food is held at a relatively high storage temperature for some time. When canned foods are not adequately cooled in a commercial cannery and are stacked away in a warehouse, they may remain at a relatively high temperature for days or even for weeks with conditions favorable for development of these bacteria. The same type of spoilage may also occur when canned goods are stored in hot climates or in warehouses insufficiently insulated against heat.

Microorganisms causing deterioration in foods preserved by heat. Certain types of foods, particularly the acid fruits, are most likely to be attacked by molds, such as *Penicillium* and *Aspergillus*. These molds do not develop unless there is free oxygen present. They fail to develop in hermetically sealed jars. They bring about changes which render the material undesirable as food, although there is no evidence that they produce poisonous substances in appreciable quantities. Usually the mold is confined to the surface, but the products of its growth frequently penetrate and flavor the whole mass.

Vegetables and meats are commonly destroyed by bacteria though in some cases molds are important. The most abundant bacteria are those which have withstood heating because of the resistant character of their spores. The organisms belonging to the butyric acid group of bacteria are relatively abundant in the soil and are present on the surfaces of most vegetables. They bring about decomposition with the evolution of considerable amounts of gas. This gas may accumulate in quantities sufficient to bulge and even to break the tin in which the food is sealed. The development of such organisms renders the food unfit for use. Some bacteria have been described which bring about decomposition in vegetables and meats without the evolution of gas. They give evidence of their presence by the development of peculiar odors and flavors. Cans of food thus affected are termed "flat sours." In many cases these organisms gain entrance to the food after the container has been closed, and their presence is due to defective sealing. Certain poisonous products of decomposition of canned meats have been described.

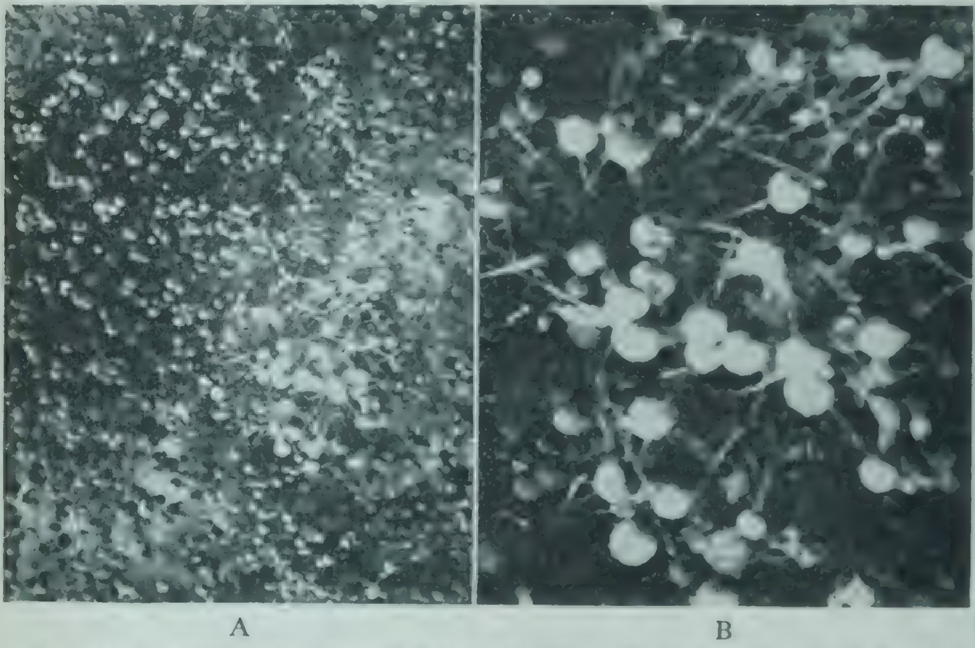


Fig. 24–3. *Aspergillus*, fruiting heads and conidiophores growing on the surface of a marmalade. A. Young and mature fruiting heads as seen under the low power of the compound microscope. B. A portion of A more highly magnified.

Preservation of Food by Cold. Practically all foods can be preserved for a time by the use of low temperatures; freezing enables them to be kept for long periods. The use of cold storage has enabled us in modern times greatly to extend the season for certain foods and to carry over surplus; it has also made possible the shipping of produce and meats for long distances.

Temperatures from 0° C. to a few degrees above are commonly used for the cold storage of fruits, vegetables, and eggs, and for the temporary preservation of meats. The normal activity of the living cells of the fruit or vegetable is greatly reduced by cold. The enzymes present act very slowly, and most (not all) microorganisms can develop little or not at all. Eggs deteriorate gradually while stored, in part due to microorganisms contained, but also probably in large measure to the autolytic enzymes present. Meats may be held for a time at these temperatures without deterioration, in fact, for a time with marked improvement in tenderness and flavor. The autolytic enzymes of the meat act slowly. Microorganisms, particularly certain of the bacteria, are not entirely inhibited from development, and in time will bring about decomposition.

Freezing and use of subfreezing temperatures for preservation of meats, fish, and poultry has long been practiced. In more recent years there has been in America a marked increase in the commercial freezing of many other types of foods, particularly fruits and vegetables. This has been stimulated by wide adoption of home refrigeration units in which frozen foods may be stored and by the development of central frozen storage lockers in which considerable supplies of frozen foods may be kept by the family to be drawn upon as needed. Frozen packaged foods are generally available in the market.

Vegetables are usually blanched before being frozen. Fruits frequently have sugar or syrup added. It is important that the food in all cases be frozen rapidly by exposure to very low temperatures and be kept constantly at subzero temperatures until used.

Most frozen foods when stored for long periods of time show some evidences of deterioration probably due to the slow action of enzymes contained.

Eggs are frozen in great quantities largely for use in the baking industry. The eggs are broken singly, inspected, and quickly frozen in bulk. These frozen eggs and those preserved by refrigeration at temperatures just above freezing make possible a much more uniform rate of consumption of eggs throughout the year.

Chemical Preservation of Foods. Substances added to or present in foods may act as preservatives in one of two ways: physically, by greatly *increasing osmotic pressure;* chemically, by *direct inhibiting action* upon microorganisms.

The chemicals most commonly used to preserve foods by increasing the *osmotic pressure* are *sodium chloride* and *sugars* (as cane sugar). Neither of these substances is actively antiseptic in low concentrations. It will be noted later that preservation of foods by drying also results in some cases in increasing the osmotic pressure of solutes. Sodium chloride is used for dry salting of fish and meats. The flesh is packed in dry salt; this rapidly removes a part of the water, and a brine is formed. Frequently this process is repeated, and the meat is packed a second time in salt. This effectually removes enough water and produces sufficient concentration of brine so that no microorganisms can develop. A brine, made of 25 per cent common salt, usually containing 10 to 15 per cent cane sugar and frequently nitrate, is used for curing meats, particularly hams. It should be noted, however, that an operation such as salting may inhibit some organisms but not others. In ham curing, for example, the salt brine is usually not so concentrated but that there is growth of certain bacteria which produce acid from the sugar and which reduce the nitrates to nitrites. The nitrite unites with hemoglobin increasing the intensity of the red color. Thick syrup and sugar are used for the preservation of fruits in jellies and marmalades. The keeping qualities of condensed and evaporated milks are in large part due to the concentration of the milk sugar, often supplemented by the addition of cane sugar. Occasionally spoilage occurs as a result of the growth of organisms which are adapted to high concentrations. Some yeasts (osmophilic types) are known which will develop in concentrated sugar solutions or syrups, such as honey.

Preservatives that inhibit the growth of organisms by their chemical action as antiseptics may be divided into two groups: those *which are produced in the food* as a result of fermentation of the food itself; and *those which are added* directly to the food.

Lactic acid formed by the action of lactic acid bacteria upon sugars may develop in sufficient quantities in certain foods to preserve these indefinitely against further change. Milk which has become sour changes thereafter but slowly, particularly if kept from contact with air. The lactic acid formed is a sufficiently powerful antiseptic so that in some countries meat is preserved by keeping it in buttermilk. Sauerkraut is prepared by shredding cabbage, adding salt, and allowing the mixture to ferment. Lactic acid is formed in sufficient quantities (usually about 1 per cent) to prevent further decomposition. The preservation of ensilage is largely due also to the lactic and other acids which are formed during the process of curing. The same is true with the fermentation of dill pickles. In alcoholic fermentation the *alcohol* produced acts in a measure as a preservative, inhibiting the growth of putrefactive and undesirable bacteria for the most part. *Acetic acid* is frequently produced also (as in vinegar) and is an efficient compound in preventing decomposition.

Among the chemicals that are added to foods for their preservation are *benzoic acid* and *salicylic acid* and their salts, *formaldehyde, boric acid,* and *borates*. *Sulfurous acid* is used in clarifying and bleaching wines and fruits. When not too concentrated it permits the growth of yeasts but inhibits the growth of undesirable bacteria in wine manufacture. The smoking of meat owes its efficiency to the presence of *creosote* and related compounds which are effective disinfectants. These destroy organisms present upon the surface and penetrate to a distance sufficient to prevent their subsequent entrance.

Preservation of Food by Drying. For every type of food material there exists a minimum of water content that will suffice for the growth of microorganisms or for the activity of enzymes. When water is present in less quantity than this, the foodstuff may be preserved for a long period of time. The exact minimum that will permit growth depends upon four factors: (1) the quantity of soluble materials; (2) the kinds of soluble materials (solutes); (3) the distribution of the moisture; and (4) the nature of the organisms or enzymes present.

Osmotic pressure is determined by the concentration of the solutes, that is, it varies directly with the number of molecules and ions in solution in a given volume. Most microorganisms can adapt themselves to a considerable variation in concentration of the solutes of the medium, but there is a limit beyond which they cannot regain their turgor. This marks the maximum concentration of solutes in which they can develop. Fruits are usually readily preserved by drying on account of their high sugar content. Raisins, for example, will keep when relatively moist, while meat or flour containing the same amount of moisture would quickly spoil.

The chemical nature of substances in solution also influences the efficacy of drying. The concentration of acid in a food by drying may be such as to produce a reaction that will totally inhibit growth through its preservative effect.

A food may contain a relatively small amount of moisture and yet undergo changes due to its uneven distribution. Commercial butter contains about 15 per cent of water, but when kept in a warm place microorganisms (principally bacteria) multiply and produce "off flavors," and even rancidity. The water is not dissolved in the fat but is dispersed through the fat in the form of globules varying in size. Conditions within these globules may be suitable for the development of bacteria which produce disagreeable flavors or aroma. Butter or butter fat entirely freed from water changes very slowly. Other foods, such as raisins or other dried fruits, may contain several times as much water without injury because of its uniform distribution through the entire food mass. The drying or curing of meat, particularly that containing considerable quantities of fat, is in part accomplished by the infiltration and saturation of the surface layers with fats and oils which form a relatively waterproof exterior.

Microorganisms vary greatly in their ability to grow in concentrated solutions. Molds may develop on the surface of jellies or preserves containing a considerable percentage of sugar; certain pseudo-yeasts or torulae develop in solutions containing 20 to 25 per cent of common salt.

It is evident from the preceding discussion that the amount of water that must be abstracted from a given food for preservation must be dependent upon the factors enumerated. In addition, a dried, or partially dried, food may be sealed from the air to prevent gross contamination and to prevent moisture being absorbed due to its hygroscopic nature. The exposure of foods to direct rays of the sun or to a high temperature in the process of drying destroys many of the organisms present. The same is true when certain disinfectants, such as smoke or the fumes of sulfur dioxide, are used in the cure.

Drying of foods may be accomplished in many ways. Exposure to the direct rays of the sun or simply to the dry air is sufficient in some climates with some foods. Artificial heat and currents of heated air are used in other instances, the temperature and rapidity of drying usually being such as will not injure the flavor. When rapid drying at low temperatures is desired, a partial vacuum may be used; this must be resorted to where the food prepared is markedly unstable. The hydraulic or other powerful press may be used to force out the excess of moisture and thoroughly compact the product. The manufacturer of cane sugar uses centrifugal action to throw off the water. Lastly the addition of salts or sugars may abstract moisture and increase the concentration of solutes.

Most food materials containing an abundance of starch are preserved by drying. Many are sufficiently dried in the natural process of ripening. Such are the grains, the flours and meals prepared from them, and the nuts. It sometimes happens through unfavorable seasons for ripening or on account of excessive moisture content of the air that molds develop on such materials and render them unfit for food. Many starchy products of the baker and manufacturer, such as biscuits, wafers, crackers, macaroni, vermicelli, and yeast cakes, are dried for preservation. Foods containing considerable quantities of sugar, particularly when acids are present, are also preserved by drying.

In dried fruits as much as 30 per cent of water may be present. Syrups, sorghum, and molasses, and jellies, jams, and preserves contain a higher percentage of sugar. Condensed milk is frequently sweetened by the addition of cane sugar, after the elimination of a considerable proportion of the water, usually by heating in a vacuum. This is not sterilized, but the concentration of sugar added is sufficient to preserve the milk. Unsweetened condensed milk, on the other hand, is sealed in cans and sterilized, otherwise putrefactive organisms would develop. Milk powder is prepared by several processes, one of which consists in forcing the milk in the form of a fine spray into a heated compartment from which the air has been partially exhausted. As it falls it dries into a fine powder which keeps well and is finding extensive use.

Foods containing a high percentage of oils and fats, such as lard, olive oil, tallow, and cottonseed oil, are relatively free from water and under these conditions will keep almost indefinitely. Butter contains about 15 per cent of water and is therefore less stable than some other fat foods. Butter fat may be preserved by melting it and removing the water. This, of course, removes at the same time the casein and milk sugar present. The deterioration of butter is dependent quite directly upon the changes produced in these substances dissolved in the water and at the interfaces between fat and water and between fat and air rather than within the fat itself.

The drying of eggs, particularly of egg white (albumen), has become a considerable industry in which various methods are in use. It has been found that egg white requires some processing before drying otherwise it deteriorates rapidly, becoming dark in color and developing a disagreeable flavor. This spoilage has been found to be due to a reaction between the glucose and certain amino groups present. Methods of stabilization of the product by removal of the glucose before drying will be discussed under processing of eggs.

The choice of a method for preservation of food will depend upon the ease with which the method can be applied to the particular food, whether or not undesirable changes in composition or palatability may be produced by the treatment, and the condition under which the food is to be subsequently kept or stored before use, particularly the temperature of storage, and the exclusion of air.

RELATIONSHIPS OF MICROORGANISMS TO FOOD PROCESSING AND TO FOOD QUALITY

The microorganisms that may be found in food may be grouped under the headings of *useful, innocuous,* and *harmful*. Microorganisms may participate in the processing of a food or a feed, they may bring about changes that are undesirable, or they may even be dangerous from the standpoint of health. In the following discussion the various important foods that are processed in whole or in part by the use of microorganisms will be considered together with the organisms that have proved useful and those which may be deleterious.

Foods and foodstuffs are rarely sterile. Microorganisms of the types of bacteria, yeasts, and molds are usually found, and occasionally even higher forms of animal life, such as the insects, trichinae, and intestinal parasites. Inasmuch as microorganisms are so constantly present in food, at least in the raw materials, their mere presence cannot condemn an article as unfit for human consumption. The kinds of organisms found and the extent to which they have multiplied are far more important.

Microorganisms may affect the acceptability of food in several ways: bacteria causing infectious diseases may be present; toxins, endotoxins, and poisonous products may be developed by the growth of other organisms; or changes affecting the palatability or nutritive value of the food may be brought about.

Pathogenic bacteria capable of producing infectious diseases may gain entrance to food by several means. With flesh food, the animal from which it came may be infected with a communicable disease, such as tuberculosis, foot and mouth disease, or the less well-defined infections due to *Salmonella enteritidis* and *S. paratyphosa*. Meat may be subjected to careless handling or be rendered infective by contact with a carrier, as in typhoid. Flies may carry pathogenic organisms to foods exposed for sale without being properly screened. It is possible that occasionally such organisms may be blown upon the surface with dust. Lack of cleanliness in the abattoir may permit one carcass to become contaminated by another, or by offal or excreta. Vegetables may be contaminated by use of night soil as fertilizer or by being washed in impure water. It is evident that the chance of transmission of disease to the human is least in those articles of food that are thoroughly cooked, as the pathogenic bacteria most likely to be present are quite certainly destroyed by exposure to the temperature of boiling water.

Organisms capable of producing poisonous toxins or endotoxins are of chief importance in foods of animal origin. The toxin of *Clostridium botulinum* is to be regarded as the product of a putrefactive anaerobe. This true toxin can poison when taken into the alimentary tract. It is readily destroyed by heat. The *Salmonella enteritidis,* on the contrary, produces a soluble endotoxin which is not readily destroyed by heat although the bacteria themselves are killed in cooking. Certain strains of *Micrococcus aureus* (*Staphylococcus*) are among the commoner causes of food poisoning. These organisms and the diseases produced will be considered later.

In the earlier literature of bacteriology there will be found much discussion of food poisoning as due to the production of *ptomaines.* These were defined as basic or alkaloidal compounds produced from proteins undergoing putrefaction. Several compounds of this type were isolated and found to be amines produced by the decarboxylation of amino acids. Such compounds are formed by bacterial action, but apparently play no role in food poisoning. The majority of the cases of "ptomaine poisoning" have been found to be due to specific infection by *Salmonella enteritidis* and *S. paratyphosa,* or to the presence of the toxins or endotoxins discussed above.

All foods, at least when exposed to contamination, sooner or later undergo changes due to microorganisms that injure them as food, at first affecting their palatability and later their nutritive value.

The participation of microorganisms in the processing of several of the foods that are plant in origin has already been discussed in Chapter 22. There are two types of food fermentation, however, that are of special importance in industrial processing. These are the fermentations that involve the production of acetic acid, and of lactic acid.

Foods from the standpoint of relationships of organisms to processing and quality may be grouped for convenience into those of plant origin and those of animal origin.

Relationships of Microorganisms to Foods of Vegetable Origin

Vegetables are most often attacked by molds, sometimes also by bacteria, causing decay and rotting. Molds belonging to the genera *Penicillium*, *Aspergillus*, and *Rhizopus* are particularly frequent. Specific disease-producing bacteria may gain entrance through use of night soil as a fertilizer in gardens as is the custom of the Chinese, or through exposure to dust and insects in the markets. Fruits may be infected through exposure or handling. They usually mold, the acidity preventing the growth of bacteria. Molds of the genus *Penicillium* are perhaps most common, producing the green mold on oranges, lemons, apples, etc. *Rhizopus* and *Mucor* are common upon the banana and small fruits or berries. Bacterial decay also occurs in nonacid fruits such as the banana. Raw food materials produced from the cereals usually have bacteria and molds. In the presence of moisture these develop, producing undesirable flavors and a marked change in the consistency. After baking into bread, etc., either bacteria or molds may produce change. *Penicillium* and *Rhizopus* are the characteristic bread molds, the former green or yellow, the latter black.

Acetic Fermentation in Food Processing. Vinegar Production. Since ancient times it has been observed that following alcoholic fermentation there appears or develops a more or less firm film upon the surface of the liquid if this is exposed to the air. This has been termed *mother-of-vinegar* and has been found associated with the production of acetic acid. The essential organisms in this film are bacteria of the genus *Acetobacter*. Many species (about fifteen) have been described, all of them rods, usually growing in chains, and not producing spores. When motile they have polar flagella, resembling in this character as well as in some others members of the genus *Pseudomonas*. Some develop only small quantities of acid and are not used in vinegar manufacture, others form acid rapidly and in considerable quantities. Some are characteristic of acetic fermentation of beer, others are from wine, and still others are important in the so-called quick vinegar process. A few of the most common species will be described briefly.

Acetobacter pasteurianus[1] produces a membrane on the surface of souring beer, at first moist, later dryer, marmorate, and finely wrinkled. It is also one of the organisms producing wine and cider vinegars. The liquid below the membrane remains clear. The organisms occur in chains; occasionally single cells become greatly elongated as filaments. The slimy membrane surrounding the cell wall is peculiar in that it is stained blue by a solution of iodine. This species, as well as some related forms, is noteworthy because of its tendency to produce great numbers of involution forms, hypertrophied cells or greatly swollen filaments. The shape and size of the cells may be varied considerably by the use of different media. The normal cells are about 1 μ by 2 μ. The organism is non-motile. It grows readily on the usual culture media, particularly wort gelatin and sugar agar. Gluconic acid ($CH_2OH(CHOH)_4COOH$) is produced from dextrose, propionic acid (C_2H_5COOH) from propyl alcohol (C_3H_7OH), and acetic acid (CH_3COOH) from ethyl alcohol (C_2H_5OH). The optimum temperature is about 34° C., the maximum 42° C., and the minimum 5° C. It does not develop in solutions containing more than 9.5 per cent of alcohol, and can produce under favorable conditions about 6 per cent of acetic acid. It is readily destroyed by the heat of pasteurization. Several other closely related species have been described from beer vinegar, differing somewhat in morphology and fermentative power.

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[1] The spelling is here changed from *pasteurianum* to *pasteurianus* to conform to the Opinion of the International Judicial Commission on Bacteriological Nomenclature to the effect that generic names ending in *bacter* should be considered as masculine and not neuter.

Acetobacter xylinoides and *A. orleanensis* have been described as the common causes of acetic acid fermentation of alcoholic solutions prepared from fruit juices such as wines and cider. Both of these produce a film over the surface of the fermenting medium. Morphologically they resemble *A. pasteurianus*. Involution forms are also relatively common, particularly in *A. orleanensis*. They grow readily upon the common laboratory media. They can produce acids from many sugars, but develop best where it is possible to oxidize alcohol to acetic acid and water. In solutions deficient in alcohol, but containing acetic acid, they carry the oxidation still farther, transforming the acetic acid into carbon dioxide and water. The cells of *A. xylinoides* are 1.2 μ by 0.8 μ, of *A. orleanensis* 1.6–2.4 μ by 0.3–0.4 μ. The optimum temperature of fermentation lies at about 30° C. The minimum probably is about 10° C.

Acetobacter schuetzenbachii (with several related species) has been described as the common cause of acetic fermentation in the so-called quick vinegar process. It grows readily upon the ordinary culture media, but does not develop a coherent film upon the surface of liquids. In this respect it differs from the forms that have been described previously. It is therefore of little significance in the production of vinegar where film formation is an essential. The cells of the organism are usually oval or elongated, not infrequently sickle-shaped or irregularly bent with rounded or pointed ends, commonly in pairs, sometimes united in chains. Considerable differences may often be noted in the size and shape of the cells in the same chain. Involution forms, particularly curved rods, are not uncommon. These organisms develop in great numbers upon the beechwood shavings used in the quick vinegar process. The cells are relatively slender, 0.3–0.4 μ by 3–6 μ and do not give the cellulose reaction with iodine. Growth is best at 25° to 30° C. In the absence of sufficient alcohol, the acetic acid formed may be oxidized to carbon dioxide and water. By the quick vinegar process, in which pure cultures of this organism have been used, acetic acid to the amount of 11.5 per cent has been obtained.

Manufacture of Vinegar. The raw materials most commonly used for the manufacture of vinegar are wine, cider, and other alcoholic fruit juices, unhopped beerwort, brandy not having too high alcohol content, the fermented juice of the sugar beet, and distilled alcohol in solution. For the most rapid fermentation it is necessary to have a solution containing 5 to 10 volumes per cent of alcohol, a temperature of 20° to 30° C., an abundance of oxygen, and suitable nutrients other than alcohol.

Vinegar, as commonly made in small quantities in the household or on the farm, is prepared from cider. This is allowed to stand in partially filled barrels or casks until spontaneous souring occurs, or the acetification may be initiated by the addition of "mother of vinegar" (a portion of the film which has developed on the surface of vinegar previously prepared), or by adding a little cider vinegar of good quality. It is necessary that a considerable surface of the liquid be exposed to the air and that the bunghole remain open to permit aeration. A film forms over the surface of the liquid, and the alcohol is changed into acetic acid. This is usually accomplished by organisms of the *A. pasteurianus* type. When the fermentation is complete, the vinegar should be drawn off, filtered, and stored in tightly stoppered vessels, as the acetic bacteria, and other organisms present, otherwise would oxidize the acid gradually and the vinegar would "lose its strength." A portion of the vinegar may be drawn off and the loss made good with fresh cider or wine, using care not to break the film. This added liquid will be rapidly converted into vinegar and the process may be repeated in three or four weeks. In wine-producing countries wine is commonly used for the making of vinegar. Cider and wine vinegar contain many organic acids, esters, etc., derived from the fruits and resulting from fermentation not found in vinegar from other sources; these determine the special aroma or bouquet of the product.

Two methods have come into general use in the commercial manufacture of vinegar, each with many local adaptations and modifications. These are the Orleans method and the quick, or German, method.

The production of vinegar by the Orleans method does not materially differ in essentials from that described above. Wine (or other alcoholic solution) is mixed with good vinegar and placed in a cask sufficiently large to give considerable surface for aeration. Fresh wine is added weekly until the cask is half filled, then a part is drawn off, filtered, and placed in vessels (casks) excluding air. The process of adding wine at intervals is continued, and vinegar is drawn off from time to time. The method was considerably modified by the work of Pasteur. Vats are now employed instead of casks, and a wooden frame or lattice floats on the surface to keep the surface film intact when it has once developed. The rapidity of acetification is directly proportional to the ratio of the area of the surface to the volume of the liquid, hence these vats are shallow. Wine (or cider, etc.) is added daily, and an equal amount of vinegar is removed.

The rapid or German method had its beginnings in the first half of the eighteenth century in a discovery by Boerhave. He found that vinegar could be produced quickly by allowing wine to trickle through a tall cask very loosely packed with pomace (the residue of the grape after the juice is expressed). This quickly sours, and the wine, as it passes down through the cask, is spread out in a thin layer over a considerable area. Optimum conditions are thus secured for rapid oxidation, and by the time the liquid has filtered through the mass (or at least after several passages), the alcohol has been changed to acetic acid, the wine to vinegar. Schützenbach, in 1823, modified the method. He sought to obtain the maximum opportunity for the liquid to come in contact with the air. His method in all essentials is used extensively today. Tall wooden cylinders are partly filled with beechwood shavings that have been thoroughly washed and inoculated with acetic bacteria by soaking in good vinegar or by pure cultures. Beechwood (sometimes bundles of rattan) is chosen, as it does not impart disagreeable or undesirable flavors to the product. By means of an automatic sprinkling device, the alcoholic solution to be acetified is distributed uniformly over the top and trickles down over the surfaces of the shavings. This exposes the liquid in thin layers to the air. Oxidizing bacteria such as *Acetobacter orleanensis* and *A. schuetzenbachii* here find optimum conditions for development and soon coat the shavings. The alcohol is rapidly converted to acetic acid. Air is admitted through openings in the side of the cylinder; the process of oxidation creates sufficient heat so that there is a constant current of air entering these openings and passing upwards. An abundance of oxygen is thus supplied. Wines and similar liquids containing much material in suspension must be clarified before use. Probably distilled alcohol, with the addition of fruit juices or chemicals to furnish nutrients other than alcohol for the organisms concerned, is most commonly used as raw material. Many of the vinegars prepared in this manner are colored by caramel to resemble cider or wine vinegars, but they do not contain the acids and extractives of the fruits characteristic of the best vinegar.

Lactic Acid Fermentation in Food Processing. One of the commonest spontaneous fermentations occurring in sugar-containing nonacid materials is the formation of lactic acid. This acid is, on the one hand, a food in itself, and, on the other, an excellent preservative. Among the foods and feeds commonly processed by use of lactic fermentation are sauerkraut, ensilage (silage), borsch, and dill pickles.

Sauerkraut. The cabbage is cleaned, shredded, bruised by tamping, and mixed thoroughly with about 2 per cent of salt as it is packed in a barrel or earthenware vessel. The surface is weighted. The salt dissolves in the sap expressed by the pressure, and by osmosis draws from the cells of the leaves a considerable proportion of their water. The cells of the leaf respire for a time, rapidly utilizing any oxygen present and creating anaerobic conditions. The leaves shrink in size as the water leaves them. Brine soon fills completely all interstices and contains in solution various sugars and nitrogenous materials derived from the leaves. A spontaneous lactic acid fermentation follows, the organisms responsible having been introduced with the leaves. Probably some of the leaf enzymes play a part in evoking the chemical changes. Usually about 1 per cent of lactic acid is formed. Many other substances are developed, some of them contributing to the desired flavor in the product. In the preparation of sauerkraut a large proportion of the antiscorbutic vitamins are preserved. Even in the early part of the eighteenth century it was urged that all ships carry a quantity of sauerkraut and that some be supplied daily to all members of the crew to prevent scurvy.

Many organisms besides the lactic acid bacteria may play a part in this fermentation. Yeasts may form some alcohol and carbon dioxide; this, however, is soon stopped by the development of the lactic acid bacteria, usually of the genera *Leuconostoc* and *Lactobacillus*. *Oospora lactis* and possibly other molds may grow on the surface of the kraut and, if the material is not protected from the air, will gradually oxidize the lactic acid and permit putrefactive organisms to develop. Occasionally the material fails to undergo normal fermentation and is spoiled by growth of the butyric acid bacteria.

Lactic Acid Fermentation of Other Foods. Many other vegetables, such as green beans and corn, may be fermented and preserved in the same manner as sauerkraut. Dill pickles are prepared by washing cucumbers, packing them in a cask with alternating layers of dill leaves, covering with water, and adding salt, spices, and sometimes sugar. The soluble sugars of the cucumber gradually diffuse out and are fermented with production of lactic acid. This accumulates in sufficient quantities to inhibit the growth of putrefactive bacteria. The Russians produce "barszcz" (or "borsch"), composed of red beets that have undergone a lactic fermentation.

The preparation of sour dough bread already discussed is based upon lactic acid fermentation by bacteria, probably largely organisms of the genus *Lactobacillus*. A certain amount of lactic acid fermentation is sought in the preparation of some beers. This is true in the so-called ginger beer discussed under alcoholic fermentation. Formerly a lactic acid fermentation constituted an early step in the preparation of tomatoes for ketchup.

In some cases lactic acid fermentation occurs in food products where it is undesirable, for example, *Lactobacillus trichodes* produces spoilage in certain wines having an alcohol content of 20 per cent.

Silage Processing. The ability of microorganisms to produce acids, particularly lactic acid, may be utilized also for the processing and preservation of feeds for animals in the preparation of ensilage (silage). As raw material many forage crops, sugar cane, sorghum, corn (maize), and potatoes have been used.

The material to be ensiled is chopped into small pieces and compacted in a silo. A silo may be a pit in the ground or a large tight container. In the United States a silo is usually cylindric in shape and constructed of wood, tile, or concrete. Care is taken to make the structure as airtight and watertight as possible, for the best conditions for silage production require the exclusion of air.

The silage should be compacted as far as possible so that no air pockets remain. It is necessary that the material ensiled contain some sugar. Corn and sorghum will have an abundance usually; to other materials as grass or legumes it may be necessary to add sugar, usually in the form of molasses ("black-strap") or occasionally whey. The respiration of the plant cells continues for a time, and the oxygen of the occluded air is rapidly taken up and replaced by carbon dioxide. Various aerobic bacteria participate in this change. If considerable amounts of air are present, oxidative changes will be rapid and the silage will heat. In properly prepared silage this heating is kept to a minimum, except perhaps in the silage near the top into which air may diffuse. There may be some slight alcoholic fermentation followed by acetification in which process the oxygen is consumed. Soon the lactic acid bacteria assume the ascendancy and rapidly convert the sugar. Occasionally, if there is excess sugar, as may be the case with sweet sorghum, alcoholic fermentation may continue after cessation of lactic acid production. In silage preparation the fermentation is effectuated without much loss of carotin and vitamin A. The lactic acid is a food substance; in fact we shall see in the discussion of digestion in the ruminant that most of the carbohydrates present in the feed are converted into organic acids before use by the body.

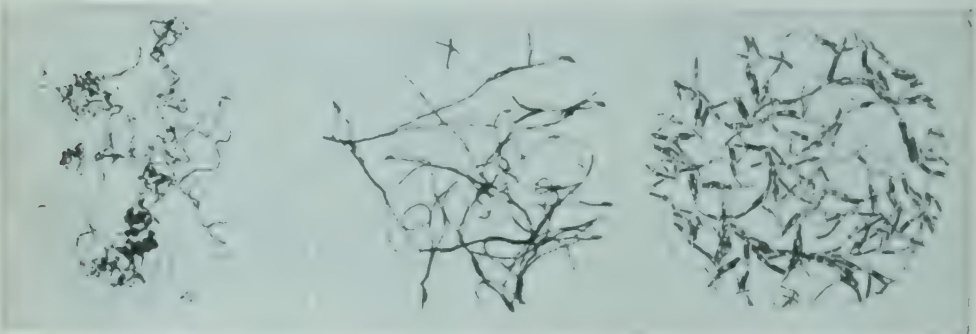

Fig. 24–4. *Lactobacillus trichodes*, producing an abnormal fermentation in wines. The morphology varies greatly with age and environment. (Courtesy of California Agricultural Experiment Station.)

The bacteria which acidify the silage are in the beginning the lactic cocci which are supplanted by species of the genus *Lactobacillus* which can grow in higher hydrogen ion concentrations and which generally produce enough acid to bring the pH below 3.0, with stoppage of the growth of all bacteria.

If air gains access to the silage, it will rapidly deteriorate due to the growth of microorganisms which oxidize the acid and develop a reaction in which decay-producing organisms may be active. If lactic acid production for any reason is not prompt, butyric acid clostridia develop with formation of bad odors and deterioration of quality. Good silage is silage which sours quickly.

Efforts have been made to expedite the development of the desirable lactic acid by the inoculation of the fresh silage with bacteria capable of producing a desirable fermentation. This has not proved particularly helpful—apparently the right kinds of bacteria are usually present.

For prevention of the growth of deleterious bacteria such as the butyric acid forms it is necessary that the silage quite promptly develop a pH of 4 or lower. Virtanen in Finland, where grass silage is important, suggested that the correct pH be impressed upon the silage by the addition, while the silo is being filled, of a mixture of hydrochloric and sulfuric acids. Silage thus treated will be preserved satisfactorily, and bacterial growth will be inhibited. The silage thus pickled has proved to be nutritious and reasonably satisfactory. This was named by its originator the A.I.V. method of silage preservation. It has been suggested that phosphoric acid constitutes a more satisfactory amendment when available. The phosphorus thus added eventually appears in the manure to the advantage of the land on which it is placed as fertilizer.

Orla-Jensen (1948) and co-workers have found that the presence of manganese salts in the medium in which the lactobacilli, important in silage production, are grown markedly stimulates the initial growth of the lactobacilli, and that beet sugar molasses contained this element in amounts that proved highly stimulating to fermentation.

Relationships of Microorganisms to Milk and Milk Products

Milk is an extremely complex mixture. It usually contains about 87 per cent of water and about 13 per cent of solids. Of the latter there is an average of about 4 per cent fat and 9 per cent of solids other than fat. The latter consists of protein (nitrogenous compounds) about 3.3 per cent, of lactose about 5 per cent, and of ash about 0.7

per cent. The nitrogenous compounds include caseinogen[2] and albumen. There is usually about four times as much caseinogen as all the other protein constituents of milk taken together. In addition there are hydrolytic enzymes, among them galactase, a proteolytic enzyme, and oxidases. Milk from the standpoint of composition is a favorable culture medium for the development of bacteria. It is ordinarily impracticable to secure milk entirely free from organisms, and the changes which they bring about may therefore in a sense be considered as normal.

Changes Occurring in Normal Milk. The changes which occur in milk may be divided into several stages: the first is the stage of bactericidal action; second, the stage of the development of lactic acid; third, the neutralization of the lactic acid; and fourth, decomposition of the proteins or putrefaction. In addition to these changes which may be regarded as constituting the common or normal cycle, it is sometimes found that milk undergoes sweet curdling or may become ropy, soapy, or colored.

The Germicidal Action of Milk. Freshly drawn milk has been shown to have a slight but definite germicidal action. The number of bacteria present in such milk is found to decrease for a time. The length of time which this germicidal action lasts differs with the sample of milk, with the numbers and kinds of bacteria, and with the conditions under which the milk is kept. At the end of a few hours, at most, the remaining bacteria begin to multiply rapidly. Various explanations have been given for this germicidal action. Some have argued that this reduction in numbers is only seeming, not real, that it is due to the agglutinating action of the milk upon the organisms present so that bacterial clumps instead of isolated bacteria develop into colonies and are counted when the milk is plated out. Others have maintained that the action is simply a differential one, that all the bacteria which first gain entrance to milk do not find a congenial environment for development, and that the forms not favored by milk die off more rapidly than the organisms which can develop in milk and increase in numbers from the first. It seems probable, however, that there is a certain amount of true germicidal action on the part of the milk. Antibodies of various kinds, such as hemolysins and

[2] Two names are used currently for the principal protein of milk, *casein* and *caseinogen*. The name casein is derived from the Latin *caseus* meaning *cheese*. In making cheese the protein of the milk is transformed into cheese protein. For the milk protein some investigators use the name caseinogen, others casein. For the cheese protein the first group uses casein, the other group paracasein.

bacteriolysins, have been determined in the milk of animals, also in the colostrum. The blood serum of the cow, as well as most of the body fluids, contain some germicidal substances, and it is to be expected that they will be given off with the milk. In addition, milk usually contains considerable numbers of leucocytes, and these do not cease action immediately after the milk is drawn, but have been found capable of ingesting bacteria for some time.

This bactericidal property does not long persist. When the milk is kept in a warm place, it disappears within a few hours. It is more persistent when the milk is kept cool. In no case does it lead to a complete sterilization of the milk. It seems probable that the germicidal action of milk is to a certain degree specific; that is, some bacteria are destroyed while others are not affected. This property is destroyed by heating milk above 80° C. It is evident that no dependence can be put upon the bactericidal action of milk for the destruction of bacteria. As stated by Rosenau and McCoy,[3] "it cannot take the place of cleanliness and ice, but may be taken advantage of in good dairy methods."

The Souring of Milk. The different types of lactic acid bacteria develop rapidly, particularly if milk is kept in a warm place. By the time 0.4 per cent lactic acid has formed, the milk tastes decidedly sour. This change is usually due to the growth of *Streptococcus lactis*. When the acidity reaches about 0.75 to 0.8 per cent, the milk usually curdles. The lactic acid bacteria, with the exception of those belonging to the genus *Lactobacillus,* rarely produce more than 1.25 per cent of acid.

Neutralization of the Acid. Sour milk may be kept under anaerobic conditions for a considerable time without marked change in its composition. Whenever exposed to the air, however, certain molds, particularly *Geotrichum candidum* (*Oospora lactis*) develop on the surface and utilize the lactic acid, oxidizing it largely to carbon dioxide and water. This results in the disappearance of the acid reaction. In part, the acid may also be neutralized by combination with the caseinogen of the milk.

Decay or Putrefaction. As soon as the excess of the acidity of the milk has disappeared, putrefactive bacteria of different kinds may develop and bring about a rapid decomposition of the milk, particularly of the caseinogen.

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[3] Bulletin 56, Hygienic Laboratory, U.S. Public Health and Marine Hospital Service, p. 487.

Unusual Changes in Milk. Milk which is heavily inoculated with organisms of the *Bacillus subtilis* group (and some other forms) may not sour normally, the lactic acid bacteria being overgrown. These organisms produce a rennet-like enzyme which causes a coagulation or sweet curdling of the milk. Later the curd is more or less completely digested.

Organisms responsible for the development of ropy milk by the formation of gums from the carbohydrates and of mucin-like substances from the proteins have already been noted. Bacteria have also been described which are capable of producing red, yellow, blue, and even black milk. Other organisms are known which produce undesirable flavors variously characterized as soapy and bitter milks.

How Bacteria Gain Entrance to Milk. *Initial Contamination.* The tissues of a normal healthy animal are usually free from bacteria. This is true of the tissues of the mammary gland. Bacteria are, therefore, not ordinarily present in the milk as it is being secreted. As it passes down through the milk ducts, it usually comes in contact with some bacteria. Certain forms seem to develop within the milk cistern and within the larger milk ducts. The first milk drawn from the teat, therefore, usually contains more bacteria than that which is drawn later, but the numbers are not sufficiently large to make any marked difference in the total number of bacteria present in the milk of the entire milking. Usually milk as drawn from the udder contains less than 100 bacteria to the cubic centimeter, although in some cows which seem to be perfectly normal, there may be larger numbers. Milk from inflamed udders may contain large numbers of bacteria. These organisms may be pathogenic. They will be discussed later.

Contamination from Hair and Skin. Unless the animal has been carefully groomed and the hair and skin of the udder and adjacent body surfaces thoroughly moistened, there will be a continual rain of dust particles and bacteria from these surfaces into the milk drawn into an open vessel. This probably constitutes one of the most important sources of microorganisms in milk. Considerable differences in the number of bacteria may be demonstrated in milk taken from animals which have been carefully cleaned and those which are dry and dirty. These organisms are largely of fecal origin.

Contamination from the Air. Dust floating in the air of the building in which the cows are milked may be a fertile source of infection of the milk. This is particularly true when dusty fodder or bedding is used. Bacteria from this source are usually of the *B. subtilis* and putrefactive types.

Contamination from the Hands of the Milker. Unless the hands of the milker are carefully cleaned, there is usually plenty of opportunity for infection of the milk from this source. From a sanitary standpoint this is particularly objectionable, as the organisms derived from this source are more likely to be capable of producing disease in man than those which have come from the animal.

Contamination from Milking Utensils. The use of the milking machine usually safeguards the milk from entrance of bacteria from the air and skin, but the teat cups, tubes, and all other parts must be scrupulously cleaned and sterilized or they will constitute a breeding ground for bacteria. Chlorine-containing disinfectants are widely used. Care is required to cleanse all milking utensils from microorganisms. Particles of rust and imperfectly soldered joints may harbor myriads of bacteria. All metal milking utensils should be thoroughly scalded; preferably the entire vessel should be heated to the boiling point of water to destroy the organisms present. In many cases milking utensils contribute a larger proportion of the microorganisms than any other source.

Contamination Due to Carelessness in Handling. There frequently is ample opportunity for infection of the milk after it has been drawn from the animal. It may be allowed to stand in open cans, or unclean dippers or other dairy utensils may be thrust into it. The water used in rinsing milk vessels may be contaminated.

Factors Determining the Numbers of Bacteria in Milk. The number of bacteria present in a given sample of milk depends upon several factors, the most important of these being the *initial contamination,* the *time* which has elapsed since the milk was drawn, the *temperature* at which the milk has been held, the care with which it has been *handled,* and whether or not it has been subjected to heat as in *pasteurization.*

The factors determining the initial contamination have already been discussed. It is self-evident that to secure a high quality of milk this contamination of the milk at the time it is drawn should be kept at a minimum. The time that elapses before milk is consumed is important, as the longer it is kept, in general, the greater will be the number of organisms present in it. Milk is a natural culture medium for bacteria, and they multiply until relatively enormous numbers are found. Important also is the temperature at which the milk is held. The acid-producing bacteria, and most of the other forms which gain access to milk, grow very slowly, if at all, at low temperatures. Milk should, therefore, be cooled as soon as possible after it has been drawn from the animal. Milk which has been properly drawn and cooled at once will keep for several days, in some cases even for weeks, without noticeable deterioration. Usually, however, certain organisms capable of multiplication at these low temperatures are present, and the milk spoils within a few days. On the other hand, milk which is not promptly cooled and which is kept at room temperatures or above quickly sours, usually in less than twenty-four hours. Pasteurization greatly reduces the numbers of bacteria present in milk.

Transmission of Disease by Milk. Among the more important infections transmitted by milk are the various diarrheas and dysenteries of infants. It has been shown that a betterment in the general character of a milk supply of a community or city is generally associated with a decrease in the death rate of infants, particularly those under one year of age. The intestinal tract of the infant seems to be particularly susceptible to infection by microorganisms belonging to the enteritidis, paratyphoid, and dysentery groups. A considerable proportion of the so-called summer complaints of infants are due to the use of milk containing such organisms. Pasteurization of the milk used for infant feeding is to be advocated wherever it is impossible to secure a milk which is certainly free from such contamination.

Typhoid fever epidemics have been traced in a considerable number of cases to infection through the milk supply. Usually such epidemics are easily recognized and differentiated from those produced by contaminated water supplies by the fact that a large proportion of the victims are children, which is not generally true of a water epidemic and the fact that the disease is generally confined to families receiving milk from a common source. The typhoid bacillus does not produce disease in animals, hence it gains entrance to the milk only through careless handling, through the use of contaminated water in the cleansing of vessels, or more commonly by the employment of one about the dairy or in milking who has "walking typhoid" or is a "carrier."

Scarlet fever and diphtheria have also been traced to contaminated milk supplies in several cases, but infections of this type are not as common as those of typhoid. Like typhoid, these diseases do not attack the cow, hence the milk is usually contaminated by some individual who has handled it.

A severe type of sore throat (septic sore throat) caused by one of the streptococci associated with mastitis (infection of the udder) has sometimes occurred in epidemic form.

The milk from a cow having tuberculosis may or may not contain the *Mycobacterium tuberculosis*. Only a small proportion of the udders of cows having tuberculosis are infected with the disease, but a considerable proportion of such animals constantly pass off tubercle bacilli. The use of infected milk is the cause of a considerable percentage of the cases of tuberculosis in children. Milk should come from herds which are tuberculin tested and from which all tuberculous animals have been removed. When this proves impossible, milk should be pasteurized, as this when properly carried out is effective in destruction of the tubercle bacilli.

The disease contagious abortion of the cow is caused by *Brucella abortus,* which is very closely related to that which causes undulant (sometimes termed Malta) fever in man. Some cases of undulant fever in man originate from milk from infected cows.

Classification of Market Milk. Market milk has been classified in certain cities into certified milk, inspected milk, pasteurized milk, and uninspected milk. The term *certified milk* originally was used to indicate a milk produced by a dairy which was regularly inspected by some health board or committee of physicians. The conditions for the production of such milk were rigidly laid down and bacterial standards for the milk were adhered to. The animals from which certified milk is secured must be free from contagious or infectious diseases; the attendants must be in good health; the stables must be sanitary, well lighted, and free from dust; the milking vessels must be sterile, and every precaution must be used to prevent the access of microorganisms to the milk. The milk must be cooled quickly after milking, sealed in bottles, and kept cold until delivery. In most cities where certified milk is inspected, it must not contain more than 10,000 bacteria to the cubic centimeter, in a few cities not more than 25,000 to 35,000. Such milk naturally sells at a higher price than ordinary market milk.

An *inspected milk* is one which comes from cows that are tuberculin tested and which is drawn and cared for under sanitary conditions, but where the extreme precautions used in the production of certified milk are not carried out.

Milk which is not certified should be pasteurized. This heating is to be followed by a rapid chilling. It has for its object the destruction of harmful bacteria and their products.

The standards for milk examination set by the Public Health Service of the United States and the increasing adoption of the standard milk inspection ordinance developed as the result of the work of a national committee has rapidly increased the use of pasteurization in safeguarding milk supplies. In most cities and many other areas pasteurization accompanied by some adequate inspection has become a legal requirement for all except special grades of certified milk. Of milk sold in cities of more than 75,000 more than 85 per cent is pasteurized.

Objections to pasteurization of milk have been raised on several counts. It has been assumed that pasteurization would kill off the lactic acid bacteria present, leaving the putrefactive organisms not killed a better opportunity to develop. This reasoning has proved to be fallacious. It has been urged that if all milk is pasteurized there would not be the same incentive on the farm to produce the milk under sanitary conditions. The obvious answer is that there should be both pasteurization and inspection of farms. The only vitamin present in milk that is in part destroyed by pasteurization is vitamin C, which prevents scurvy. However the amount present in milk is insufficient for adequate infant nutrition, and the loss is readily made good in other foods. There is no adequate evidence that the digestibility of the pasteurized milk is impaired.

Is pasteurization of milk needed in rural districts and small towns? There is evidence that at the present there are more milk-borne epidemics outside the cities than inside. Pasteurization is desirable.

Various methods of home pasteurization have been suggested. Perhaps the simplest and one of the most satisfactory is that proposed by the Bureau of Dairy Industry of the U.S. Department of Agriculture, as follows:

> The milk may be pasteurized in regular milk bottles as follows: Remove the cover from one bottle, pour out a little of the milk, punch a hole in the cover, replace the cover and insert a thermometer. Then set all the bottles of milk on a rack in a pail filled with cold water nearly to the top level of the milk and heat until the thermometer in the milk registers 145° F. Remove the pail from the heat and leave the bottles in the hot water for 30 minutes, reheating if that is necessary to keep the temperature at

145° F. After the thirty minute period replace the hot water gradually with cold water until the milk is cooled, preferably using ice in the last water.

Commercial pasteurizers are of two types, those which pasteurize by the *flash* process or by the *holder* process. In the *flash* or continuous process the milk is heated to the required temperature (80°–85° C.) and held at that temperature for thirty seconds to a minute. It is then cooled and maintained at a low temperature until distributed. In the *holder* process the milk is kept at the temperature required (60°–65° C.) for about half an hour. It is then cooled and bottled. It has been found in practice that the holder method is the more efficient, as it destroys a larger percentage of the bacteria. Usually over 99 per cent of the bacteria present in milk are destroyed by proper pasteurization. Among those destroyed are practically all the pathogenic forms, including the tubercle bacillus.

The temperature at which milk must be pasteurized is of course determined by the thermal death point of the more resistant of the pathogenic organisms which may be present, and the one upon which attention has been particularly concentrated is *Mycobacterium tuberculosis*. The temperature of 60° C. for thirty minutes will quite certainly destroy this organism when pasteurization is properly carried out. Rosenau draws the following conclusion.[4] "So far as we may conclude from the evidence at hand, the heating of milk to 60° C. for twenty minutes destroys pathogenic microorganisms without seriously affecting its composition or quality and without sensibly hurting its food value. We have authority for the statement that milk pasteurized at 60° C. for twenty minutes is 'live' milk, rich in zymogens, and that such milk retains entirely the taste of fresh milk and is quite as digestible."

Processing of Milk and Milk Products. Milk allowed to stand for a few hours usually undergoes lactic acid fermentation. This accomplishes a certain diminution in the amount of sugar, and curdles or precipitates the casein. This fermentation is brought about almost exclusively by bacteria.

The bacteria producing lactic acid in milk may be placed in two principal groups, those that bring about little chemical change in the milk other than in the carbohydrates and in the production of acid and those that cause formation of gas or digestion of protein in addition to the acid formation. The first may be termed the normal or desirable type, the second the undesirable.

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[4] M. J. Rosenau, Bulletin 56, Hygienic Laboratory, U.S. Public Health and Marine Hospital Service, p. 651.

The True or Desirable Lactic Acid Bacteria. The organisms primarily producing lactic acid belong for the most part to the genera *Streptococcus* and *Lactobacillus.*

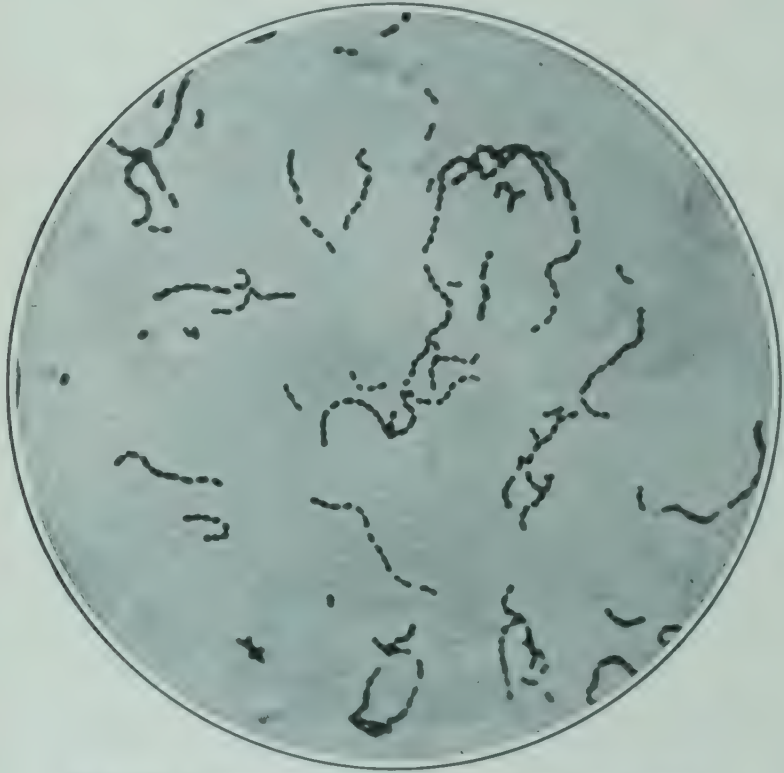

Fig. 24–5. *Streptococcus lactis,* from milk. (× 1000)

THE GENUS *Streptococcus.* There was much confusion in the older literature relating to the souring of milk. It seems now to be well established that several species of non-pathogenic streptococci are the most common organisms instrumental in bring about lactic acid fermentation of milk.

Streptococcus lactis has spherical cells (sometimes slightly elongated), occurring in chains. It is gram-positive, does not produce spores, and is non-motile. Apparently there are some strains, perhaps distinct varieties, capable of producing capsules. Some of these capsulated types are responsible occasionally for the development of ropiness in milk and for the sliminess experienced in certain cultures or starters. *S. lactis* grows well, but never luxuriantly, on many of the laboratory media. Its growth is stimulated by the presence of suitable sugars which it may ferment. Media prepared from whey are frequently found to be useful. Lactose agar plates poured from souring milk will show the organisms developing as small pinpoint colonies, frequently lying somewhat below the surface of the medium. If litmus or some other suitable indicator is present, the medium surrounding the colony will be intensely acid. Upon agar slants the colonies are usually more or less separated, or discrete, at first scarcely visible, and at last dewdrop-like in appearance. In ordinary broth the organism does not grow well unless sugar is present. Usually the medium clears rapidly by sedimentation. Gelatin is not liquefied.

The common sugars, dextrose, sucrose, and lactose, are all fermented with the production of lactic acid but never with formation of gas. There is some confusion in the literature as to the type of acid produced, but pure cultures in certain sugars at least frequently produce the dextro type only. When grown in milk, sufficient acid is usually formed from the lactose (0.5 to 1.25 per cent) so that coagulation or development of an acid curd occurs. The curd so formed is usually smooth, free from gas bubbles, has a pleasant acid flavor, and shows little or no tendency to shrink and expel the whey. Its optimum growth temperature is about blood heat, but it grows well (particularly in milk) at room temperatures and quite slowly at refrigeration temperatures. Apparently there are strains of this organism, perhaps closely related species, which can resist the ordinary temperatures used in pasteurization. This is evidenced by the fact that milk which has been pasteurized in commercial plants usually contains viable *S. lactis*.

Buttermilk owes its flavor and acidity largely to bacteria of this group. Artificial buttermilk is prepared by inoculating sweet milk with a considerable proportion of pure culture of lactic acid bacteria. When the milk has curdled it is beaten or churned so that the casein is divided into fine particles. This is to be preferred to true buttermilk as a beverage because of the greater uniformity in flavor and texture secured. Milk soured in this manner may be flavored with fruit juices and frozen into a kind of milk sherbet.

THE GENUS *Lactobacillus*. The bacteria of this genus are all gram-positive, non-spore-producing rods developing commonly in milk and various fermented foodstuffs. A few species have been reported from the alimentary tract of man and animals. The group is sometimes known as the group of *high-acid bacteria* because certain of the species will grow in a medium having a higher hydrogen ion concentration than will permit of the growth of most bacteria, even of the streptococci. This, however, is not true of all the organisms belonging to the group, for some of them do not produce quantities of acid larger than those developed by *Streptococcus lactis*. Some of the species are relatively short rods. Most of them, however, are decidedly elongated, sometimes almost filamentous. The shortest rod forms apparently intergrade with *Streptococcus;* it is accordingly sometimes difficult to differentiate between them and *S. lactis*.

One of the best-known species belonging to this group is the *Lactobacillus bulgaricus,* the organism first described by Metchnikoff. When traveling through southern Russia and the Balkan countries, this investigator was much impressed by the large quantities of fermented milk and sour milk beverages used by the peasant classes as food. He also reached the conclusion that the peasants of this region were particularly long-lived. He attributed this longevity to the large use of the soured milk in the diet. He made a study of the favorite milk beverages and succeeded in isolating the organism chiefly responsible for the lactic acid formation. Inasmuch as it was isolated from the Bulgarian soured milk it was given the specific name *L. bulgaricus*. The organism is a relatively large rod, sometimes filamentous. The cells are usually 0.5 to 1.0 μ in diameter, and 2 to 3 μ in length. In old cultures, however, some of the rods may be many microns in length. Although the organism is definitely gram-positive in young cultures, smears made from old cultures may show a mixture of gram-positive and gram-negative rods. Single cells may sometimes show a portion retaining the gram stain and another portion decolorized and taking the contrast stain. This organism does not grow very readily upon the ordinary culture media, although its development in milk at the right temperature is abundant. Colonies produced in agar resemble a tiny mass of wool. Thread-shaped rods will be seen to penetrate the medium in all directions. A stab culture in whey agar or lactose agar gives a fir tree appearance. *L. bulgaricus* grows best at temperatures above blood heat, the optimum being 42° to 45° C. Growth is slow at room temperatures. The total amount of acid formed is usually greater than that produced by the *Streptococcus lactis*, sometimes as much as 4 per cent being observed. Inasmuch as growth evidently continues in these relatively strong solutions of acid, the organism is termed acid tolerant or *aciduric*. The curd produced in milk is somewhat slimy, smooth, and does not tend to contract and expel the whey. The organism grows best under anaerobic conditions, better therefore below the surface of the medium than at the top.

Other species which belong to this group of *acid-tolerant* or *aciduric* bacteria have been isolated from the alimentary tract and feces. One of these, *Lactobacillus acidophilus,* has been used rather extensively therapeutically. When ingested with the right food it is able apparently to supplant other organisms in the colon and thereby prevent the growth of undesirable putrefactive bacteria. A related organism found in abundance in the stools of breast-fed infants is *Lactobacillus bifidus.*

Undesirable Lactic Acid Bacteria. The abnormal and undesirable lactic acid bacteria are represented by a single group, that form gas in milk.

GAS PRODUCING BACTERIA. THE GENUS *Aerobacter.* The organisms belonging to this group are of intestinal and soil origin: *Escherichia coli,* motile and without capsules; and *Aer. aerogenes,* non-motile and frequently capsulated when grown in milk. They do not produce spores and are gram-negative, differing in this latter respect from the other lactic acid bacteria.

These organisms grow readily on ordinary culture media. The colonies on agar or gelatin plates are easily differentiated from those of the preceding group because they are much larger and inclined to be slimy. The optimum growth temperature is about blood heat, but the organisms grow well at lower temperatures. In the presence of suitable carbohydrates these organisms can develop under anaerobic conditions, but when deprived of sugars they are aerobic. Many of the sugars are fermented (among them dextrose, sucrose, usually maltose and lactose) with formation of both acid and gas. The acids formed from the lactose of milk may reach 1 to 1.25 per cent, but are lactic acid only in part, acetic and other volatile acids being also present. In contrast to the normal or desirable lactic acid bacteria, they form the levolactic acid. The gas produced is a mixture of hydrogen and carbon dioxide.

The curd formed in milk by organisms of this group is more or less torn by gas bubbles; it shrinks and expels a considerable proportion of the whey. The flavor developed is quite undesirable. These organisms, particularly *Escherichia coli,* when present in great numbers in milk, indicate considerable fecal contamination. Such milk should not be used for human consumption. They also injure milk for the manufacture of cheese and produce undesirable flavors and aromas in butter made from cream soured or ripened by their action.

Microorganisms in Butter Production. The ripening of cream preparatory to churning is essentially a souring induced for the purpose of rendering churning easier and to impart certain desirable flavors and aromas to the butter. However, butter may be churned from sweet cream. The bacteria normally present in cream will usually produce the acid and other products desired, and dependence is placed upon these in the manufacture of butter in the home. In the commercial manufacture of butter in the creamery the cream is pasteurized and then inoculated with a culture of lactic acid bacteria termed a *starter*.

The flavor and aroma of butter will depend very largely upon the type of organisms which have been growing in the cream. Butter fat absorbs considerable quantities of dissolved substances present in the fermenting cream; it is, therefore, highly desirable that these should be of the proper character. If batches of cream are allowed to ripen spontaneously without the addition of any starter, there is likely to be a marked lack of uniformity in the product. A much better and more uniform result is secured by mixing the cream which is to be ripened with a considerable inoculum of desirable lactic acid bacteria. Such organisms are sold under the name of commercial starters. They are introduced into pasteurized milk and allowed to grow until the milk has reached a satisfactory state of acidity; this then is added to a larger amount of milk and finally the whole is used as starter in the cream. A heavy inoculation with the desirable microorganisms and their growth products usually overwhelms undesirable types of bacteria and leads to the development of a satisfactory product.

The changes brought about in satisfactory ripening of cream are not the result simply of the growth of *Streptococcus lactis*. Other organisms closely related to it must grow with it. Cream which has been ripened by means of a pure culture of the ordinary *Streptococcus lactis* does not produce butter with as satisfactory a flavor and aroma as that produced by cream which has been inoculated with a mixture of these organisms. The ripening produced by a starter is therefore an example of associative action. The true *Streptococcus lactis* apparently produces very little change in milk or cream other than the development of the pure lactic acid from lactose. The associative organisms, *Streptococcus citrovorus* (*Leuconostoc citrovorum*), can by its own growth bring about very little change in milk. When grown in the presence of *Streptococcus lactis,* however, the activity of this organism is greatly stimulated, and small amounts of volatile acids and other compounds are developed. It is to the absorption of these that the butter owes its characteristic aroma and flavor.

The most important of the substances contributing to a desirable butter aroma is diacetyl ($CH_3 \cdot CO \cdot CO \cdot CH_3$). This is produced indirectly by many species of bacteria, among them some of the lactic acid bacteria (as *Streptococcus citrovorus*) used in butter cultures or starters. In the butter culture, the citric acid normally present in milk is attacked with the formation of acetyl methyl carbinol or acetoin ($CH_3 \cdot CO \cdot CHOH \cdot CH_3$). This compound is readily adsorbed to the butter fat. It is gradually oxidized to diacetyl, which is volatile and when present in the right amount gives the desirable butter aroma. Under certain conditions (particularly anaerobic) the acetoin may be reduced to 2,3-butylene glycol ($CH_3 \cdot CHOH \cdot CHOH \cdot CH_3$). Apparently a desirable butter culture is one which develops enough diacetyl and acetoin to produce and maintain the desirable aroma and flavor.

Some varieties of *S. lactis,* or closely related species, form products which may be undesirable in butter or cream. One of these, *S. lactis* var. *maltigenes,* produces a malty flavor.

Microorganisms in Cheese Processing. Cheeses are divided into two classes, those in which the curd is formed by the action of lactic acid, and those in which it is developed by the action of rennet.

Acid Curd Cheese. Milk, usually whole milk, is allowed to sour spontaneously, or as a result of inoculation with starter. The curd is separated from the whey by heating, and formed into balls or cakes. In some cases, it is mixed with cream or butter before use. These constitute the so-called cottage cheeses and Dutch cheeses commonly prepared in the household. They require no ripening before they are consumed and may be regarded simply as a special form of sour milk curd. Such cheeses cannot be kept for very long periods because molds and yeasts begin to develop which soon spoil the flavor and render the cheese unfit for use.

Rennet Curd Cheeses. A great variety of cheeses is produced by the action of rennet upon milk. A dozen or more are commonly sold on the market in the United States, and several hundred in addition have been described. Several factors contribute to the differences in the character of these cheeses: the source of the milk, i.e., cow, goat, and sheep; the preliminary treatment of the milk, as homogenization; the amount of moisture retained in the curd; the amount of salt; the nature of certain condiments added; the size of the cheese cakes; the temperature and other conditions of ripening; the presence of certain specific organisms, particularly molds and bacteria, in some cases intentionally added and in others normally present under the conditions of manufacture.

The cheeses produced by the action of rennet may be divided into two classes, the *hard* and the *soft*. The primary difference between these classes is the amount of whey or moisture left in the curd with the consequent variations in the ripening process.

One of the commonest hard cheeses is *Cheddar cheese*. The milk used in the production of this cheese must be of good quality. Particular care must be used that gas-forming bacteria of the *Escherichia coli* and *Aerobacter aerogenes* types do not develop. Some acid is usually allowed to form before the rennet is added, commonly about 0.2 per cent. Rennet is then added to the milk, which quickly curdles. The curd is cut into small pieces by means of knives; it then shrinks and expels the whey. Acid-producing bacteria continue to develop. The acid unites in part with the casein; the latter changes in consistency somewhat, and the separate pieces of curd unite to form a coherent mass. The whey is largely removed by pressing the curd into cakes of different sizes. The cheese is then placed in a curing room and allowed to remain until the ripening process is complete. Some pressure is felt by cheesemakers to make cheese from pasteurized milk. Several difficulties have been encountered, one of the most important being the development of bacteriophage which interferes with the normal souring and ripening of the milk and to some extent with the after ripening.

This ripening process is probably due to changes brought about by a combination of action of several agencies. Normal milk contains a proteolytic enzyme (a proteinase). Rennet added to the milk contains more or less pepsin, which is also a proteolytic enzyme. Certain *bacteria* may also secrete proteolytic enzymes, though their importance has been brought into question. In the sequence of changes that occur during the ripening process, probably the first is production of lactic acid by bacteria. These continue to multiply until the milk sugar has all been used up. The lactic acid formed combines with the casein. The proteinases and pepsin continue their action. The pepsin is effective only in the presence of acids, hence their development creates favorable conditions. It is not entirely certain whether bacterial enzymes are responsible in this cheese for any of the proteolysis, but such is not impossible although no species has thus far been found that can satisfactorily duplicate in pure culture the changes which take place in the cheese itself. Proteolysis or digestion of the casein results in rendering the curd relatively soluble. At the same time, flavoring substances, the nature of which is not thoroughly understood, are developed. In some cases, these are in part ammonia and related compounds, and part the ethyl esters of the fatty acids, particularly the volatile acids. It is possible that the autolytic enzymes liberated by the death of the lactic acid bacteria may also assist in the development of flavor.

When bacteria belonging to certain species of the genus *Aerobacter* are present in considerable numbers, gas forms in the curd, and malodorous compounds develop. These organisms are generally present in small numbers in milk, and cheese usually contains some gas spaces, but these are not numerous. Yeasts which can ferment lactose with gas production have also been described from cheese.

Swiss cheese is characterized in appearance by the presence of small gas pockets or "eyes" formed as the result of the activity of bacteria, probably in part at least those belonging to the genus *Propionibacterium.*

Camembert cheese may be taken as a type of soft cheese. The curd is produced as has been described for Cheddar cheese, but is allowed to drain without being cut up or heated, thus retaining a much larger proportion of the whey. The cheese is molded into small cakes and placed in a curing room, the temperature of which is usually not very low. Molds soon cover the surface of the cheese, *Geotrichum candidum* (*Oospora lactis*) developing first, and later various species of *Penicillium,* particularly *P. camembertii.* These molds are capable of utilizing organic acids as food. The acidity of the cheese is thus gradually reduced, and at the same time the molds secrete proteolytic enzymes which gradually diffuse toward the interior of the cheese. The mycelium, however, ordinarily does not penetrate the cheese to any considerable distance. When the proteolysis occasioned by this mold development has penetrated to the center of the cheese, ripening is completed. The reduction in acidity may allow the development of putrefactive bacteria and consequent spoilage, although in most cases the antibiotics produced by the mold inhibit bacterial development efficiently. It seems probable that the molds in this case are much more important than the enzymes ordinarily present in the cheese. It is believed that the proteolytic changes in the texture of this cheese ripening are due to the *Penicillium*, while the characteristic flavor has been ascribed to the *Oospora* (*Geotrichum*).

Roquefort cheese is probably the best-known cheese made from sheep's milk. The cut surface of this cheese shows a characteristic greenish blue mottling due to the presence of a specific mold, *Penicillium roquefortii*. The cheese is inoculated with this organism by allowing bread to mold and mixing the moldy bread crumbs with the curd.

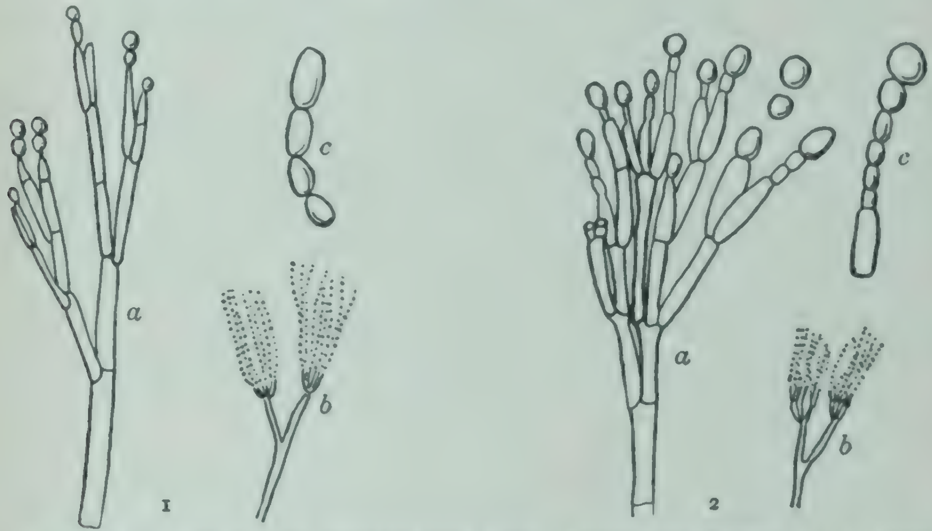

Fig. 24–6. 1, *Penicillium camembertii* from Camembert cheese. 2, *P. roquefortii* from Roquefort cheese. *a*, characteristic branching of the conidiophores; *b*, same on smaller scale; *c*, conidia. (Adapted from Thom.)

Penicillium is an aerobic organism, so to facilitate its growth within the cheese, holes are punched by means of stiff wires. Somewhat similar blue cheeses are also made from cow's milk, such as Danish blue cheese and Wensleydale. It has been found that the characteristic aroma and flavor of Roquefort and similar blue cheese is due to the production from the milk fat of the compound methyl amyl ketone. Apparently one reason for the superiority of sheep's milk in making the blue cheese is that the fat globules are much smaller than are those of cow's milk. The smaller the globules in milk of a definite fat content the greater the surface area and the better the opportunity for the fat to be attacked by the mold enzymes. Hammer and his associates discovered that by the process of homogenization the fat globules of cow's milk may be finely subdivided and that such homogenized milk constitutes a satisfactory substitute for sheep's milk in manufacture of a blue cheese of the Roquefort type. This discovery has been the basis for a considerable industrial development.

Relationship of Microorganisms to Changes in Ripening and Curing of Meats

The flesh of certain animals is commonly subjected to a more or less protracted ripening process before it is used as food. With the changes that occur during this process microorganisms normally have little or nothing to do. By the time the muscle tissue has cooled, or even before, the condition known as *rigor mortis* sets in. This is a stiffening of the tissues due to two factors: in part, to the more or less complete solidification of the fat, but principally to the coagulation of the muscle protein, that is, the transformation of the myosinogen into myosin. Authorities are not entirely agreed upon the cause of the latter change. Lactic, and some other acids, are formed in the muscles, probably as a result of the continued metabolism of the cells after the death of the animal. An autolytic enzyme seems to be freed upon the death of the cell. It is related in action to the lab ferment or rennin. The combined action of acid and enzyme is to coagulate the muscle protein. The flesh thereafter gradually becomes more tender, possibly more digestible. This is probably due to the continued action of the proteolytic autolytic enzymes of the cells. This may be demonstrated experimentally by the removal of a portion of tissue, or an entire organ, such as a liver or spleen, under aseptic precautions to prevent bacterial infection and activity. When kept in physiological salt solution, such a tissue undergoes autolysis, and in the course of time becomes more or less completely digested. Such an autolysis, only partial, is the essential transformation in the ripening of the flesh foods. In a certain degree, this seems to be desirable. It is sometimes difficult, however, to differentiate between this normal change and putrefactive changes brought about by microorganisms.

Microorganisms are sometimes active in the process of meat curing. Hams and bacon are frequently cured in a brine which includes salt, sugar, and sodium nitrate (saltpeter). Certain bacteria produce acid and reduce the nitrates to nitrites. The nitrite ion combines with hemoglobin of the red blood cells and similar iron-containing constituents of the cells, producing the much brighter red color desired in the cured product.

Microorganisms and Eggs

Eggs rapidly decompose when stored at unsuitable temperatures. The initial infection of the egg with bacteria may take place while the egg is still within the oviduct of the hen, or organisms may gain entrance after the egg is laid. Egg white has been shown to possess distinct antiseptic properties due to the presence of a lysozyme. Many species of bacteria are quickly destroyed when mixed with it. This is not true of the yolk, for this is a favorable growth medium for many species of bacteria. It is not probable that this bactericidal property of egg white persists indefinitely, but it is doubtless responsible for the fact that the egg keeps as well as it does. The higher content of CO_2 in the fresh egg is also apparently related in some way to its keeping quality. Many species of organisms, both bacteria and molds, have been isolated from decaying eggs. Probably some of the changes that take place in stored eggs are autolytic in nature.

Enormous numbers of eggs are frozen or dried. The principal problem in freezing is to prevent the inclusion of eggs that have a high bacterial count or that have begun to spoil.

Processing of eggs, particularly egg white in drying, constitutes a special problem because of the glucose content. When dried, the glucose and amino acid radicals of the protein combine and produce a brown to black pigment which discolors the product and renders it unpalatable. This is avoided in practice by subjecting the egg white to a fermentation which will remove the sugar. Yeasts have been used, but usually a spontaneous fermentation occurs which is usually adequate.

The fact that salmonellosis of the domestic fowl, caused by *Salmonella pullorum*, may be transmitted through the egg to chicks has led to several studies to determine the occurrence of bacteria of the genus *Salmonella* in dried eggs. There have been reports of isolations. The extent to which this would constitute a health hazard is uncertain. The fact that a single infected egg might during drying or freezing be mixed with large numbers of other eggs makes these processed products somewhat more suspect than shell eggs.

CHAPTER 25

Microorganisms in Water and Sewage

THE FLORA OF RELATIVELY PURE WATER—NORMAL WATER BACTERIA

Sterile water is rarely found in nature, and then usually under somewhat exceptional conditions such as that in boiling springs. Water obtained even from deep wells and from springs usually contains a few bacteria. Surface waters, those of streams, ponds, lakes, and the water of the ocean, always contain bacteria, usually in considerable numbers. Even the waters of salt and alkaline lakes have an abundant and characteristic bacterial flora, as do those of sulfur springs. The organisms thus present under natural conditions may be designated as the normal water flora as they are constantly found and in no case are they capable of producing disease in man. For the most part, they are organisms that grow best at the temperature of the water in which they are found. A few of the organisms found quite commonly in surface waters may be noted.

Cocci are quite common. They are often chromogenic, either micrococci or sarcinae. Probably the commonest of these is *Sarcina lutea,* which develops lemon yellow colonies when grown upon artificial media; and *Sarcina aurantiaca,* with a golden yellow pigment, is also common, as are other cocci producing pink, red, yellow, and orange pigments. Certain non-chromogenic cocci, as *Micrococcus candicans,* may also be found.

Chromogenic rods such as *Serratia marcescens* (red pigment), *Flavobacterium aurantiacum* (orange pigment), and *Chromobacterium violaceum* (violet pigment) are common in surface waters. Polar flagellated bacteria, as *Pseudomonas fluorescens* which forms fluorescent colonies upon media, are usually abundant. Water derived from surface drainage may have many of the normal soil bacteria such as members of the *Bacillus subtilis* group. Some special groups of bacteria, such as the *Caulobacterales* which adhere to surfaces by gelatinous stalks, are commonly present, and particularly in sea water certain light-producing (photogenic) bacteria such as *Pseudomonas phosphorescens*. Surface waters usually contain other living organisms such as algae, higher plants, and insects; it is generally possible to find bacteria capable of destroying or digesting the dead bodies of these organisms. Among these may be noted bacteria which can decompose the cellulose of plant walls, the agar of the walls of certain marine algae, and the chitin of insects and crustacea.

A potable water may be defined as one which contains no disease-producing bacteria and no organic or inorganic substances deleterious to health. The presence of considerable quantities of organic matter leads to the multiplication of many putrefactive bacteria, such as the spore-bearing anaerobes. While these are not necessarily injurious to health, they are undesirable.

FLORA OF IMPURE OR NON-POTABLE WATER

The organisms of importance in impure water may be either pathogenic or non-pathogenic. Among the pathogenic microorganisms which may gain entrance to water supplies through contamination with sewage are the bacteria causing typhoid fever, paratyphoid, bacillary dysentery. Asiatic cholera, the protozoon of amebic dysentery, and several viruses including that of poliomyelitis (infantile paralysis).

An impure or non-potable water may be defined as one which contains more or less sewage, one, therefore, that is not fit for human consumption primarily because of the possible presence of pathogenic microorganisms. Relatively few species of organisms are held to be indicative of sewage pollution. The most important of these are *Escherichia coli*, *Aerobacter aerogenes*, *Streptococcus pyogenes*, and *Clostridium welchii*. These organisms are quite constantly present in the intestines of man and animals. When they are found in considerable numbers in water, it may be concluded that there is contamination with sewage, inasmuch as they do not often continue to multiply outside of the body for very long periods after their elimination.

Relationship of Water to Health

A pure water supply is one of the best safeguards of the health of individuals and of communities. Relatively few diseases are actually transmitted by impure water. All enter through the alimentary tract and the organisms leave the body with the excretions.

A pure water supply, however, seems to affect the general health of a community and to decrease its death rate wholly apart from the decrease due to the elimination of the specific diseases mentioned above. It has been noted, for example, that when a city changes from a contaminated to a pure supply, there is not only a marked decrease in the typhoid death rate, but also in some instances decreases are noted in the number of deaths from tuberculosis, pneumonia, and other diseases. In practically every instance there is a decided decrease in the total death rate. This has been termed the Mills-Reincke phenomenon, after the men who first called attention to these facts. This phenomenon must be due to one of two causes. Either diseases not ordinarily regarded as being carried by water are in a certain percentage of cases so transmitted, or the use of an impure water supply so affects the average health of a community that the individuals become more readily susceptible to other diseases. It is probable that the latter is the true explanation.

Bacteriological Examination of Water. The methods used by the bacteriologist to determine the potability of water may be either quantitative or qualitative. In the former, the total number of bacteria present is determined. In the second, an effort is made to identify kinds or groups of bacteria. Both are useful and they are generally employed together.

A sample of water for bacteriological analysis must be collected carefully. It is customary to use wide-mouthed, sterile, glass-stoppered bottles. If the sample is to be taken from a well, sufficient water must be pumped for some minutes to remove that which has been standing in the pipes in order that a sample representative of the water in the well itself may be secured. If the collection is from a tap, the water should be allowed to run for a time before sampling. If it is taken from a stream or body of water, the unopened glass bottle should be thrust below the surface before removing the stopper, the bottle should be filled below the surface and the stopper returned. This avoids collecting the dust which may be present upon the surface and thus vitiating the results. When samples are taken at a depth in a body of water, various devices are used whereby a bottle or flask is lowered and automatically opened and closed at the depth required. When the sample has been collected, it should be taken to the laboratory and examined as quickly as practicable. If it is necessary to send the water for some distance, it should be packed in ice and hurried to its destination. These precautions must be taken, as the conditions under which the water is held in the bottle are quite different from those in the source from which it was secured. If the water comes from a cold spring, for example, the bacteria present begin to multiply rapidly. Within a few hours they may be hundreds or even thousands of times as numerous as in the original sample, and an examination of such water will not reveal the true condition of the original supply. The more highly contaminated the water, the more essential is a prompt examination.

Quantitative Methods of Water Examination. The number of bacteria present in a water sample is usually determined by pouring agar plates. Various dilutions of the water to be examined are prepared by means of water blanks of known volume and sterile pipettes.[1] One milliliter of each of the dilutions to be examined is placed in a sterile petri dish and a tube of liquid sterile agar is poured into this, thoroughly mixed with the water by tilting, and allowed to stand in a cool place until the medium has hardened. This effectually prevents the bacteria from moving about, and colonies develop from these bacteria entrapped in the medium. It is customary in making an analysis of this kind to prepare duplicate plates of each dilution, incubating one set at about 20° or 22° C., and the other at 37° C., as most of the water forms develop well at one or other of these temperatures. In most cases the numbers of colonies on these plates can be counted at the end of forty-eight hours. The number of colonies that has developed on a plate multiplied by its dilution should give the approximate number of organisms per milliliter present in the original sample. This, however, is true only within certain limits. When bacteria are too closely crowded on the plate, the growth of one colony may inhibit the development of others. It is customary, therefore, to count those dilutions which show between 30 and 300 colonies on the plate, because the presence of a larger number will ordinarily give too low a total count. If it is desired to identify the organisms present, representatives of each type of colony may be transferred to other artificial media and the cultural, physiological, and morphological characters may be ascertained.

It is not always easy to interpret the results of a quantitative analysis of water. It is necessary first that we know something of the number of bacteria to be expected in a sample of water that is not polluted. In general, it is true that pure waters contain small numbers of bacteria and polluted waters contain large numbers. Water from a deep well or spring may sometimes be obtained which is wholly free from microorganisms, or at the most contains no more than 10 or 20 organisms per milliliter. The water in ponds and lakes is rarely as pure as this, but when there is not an excess of organic material and no sewage pollution, the number rarely runs above 200 or 300. The same may be true of pure stream waters except during seasons of high water, when considerable amounts of surface material containing soil organisms may be washed into the stream. A polluted water is one which contains an admixture of sewage. It usually contains several thousands, sometimes even millions, of organisms per milliliter. It has been stated that water containing less than 100 bacteria per milliliter is probably safe, water containing 500 per milliliter is to be regarded as suspicious, and one containing more than 1000 organisms per milliliter is unsafe. These statements taken alone, however, do not mean much. In a final determination of potability, it is usually best to make qualitative examinations as well. In some cases, however, examinations of water by quantitative methods may prove valuable. In the larger water purification plants where the water passes through sand filters, it is customary to determine the extent of the purification of the water by periodic analyses as it leaves the filter beds. A filter when working efficiently should remove a very high percentage of the bacteria present in the original supply. It is therefore possible relatively quickly to recognize defective action of a filter by such a procedure.

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[1] A water blank is a test tube or flask containing a definite volume of usually 9 cc., 49 cc., 95 cc., or 995 cc. of sterile water. By the addition of 1 cc. of water, for example, to 9 cc. of sterile water, a dilution of 1:10 is effected. Other dilutions are prepared in a similar manner. The pipettes customarily used have a capacity of 1 cc. or 5 cc.

Qualitative Methods of Water Examination. Qualitative water examinations are made for one of two purposes, either for the isolation of specific pathogenic organisms or for the recognition of typical sewage bacteria.

Isolation of Specific Pathogens. The isolation of specific pathogenic organisms such as *Salmonella typhosa* or *Vibrio cholerae* from a contaminated water supply is difficult. There are several reasons for this. Pathogenic bacteria may occur sporadically in a water supply; it is not often that they are constantly present. Since the incubation period of typhoid fever is usually from nine to ten days, if examinations are begun upon a suspected water supply only after an epidemic of typhoid has developed, the organisms in all probability will have disappeared. Even when constantly present, they usually are relatively scarce. Even in the stools of a typhoid patient the cells of *Escherichia coli* usually outnumber those of *Salmonella typhosa*. When cultivation is attempted upon artificial media, therefore, the typhoid bacteria are usually overgrown. Successful isolation from water supplies has been accomplished by inoculation into media (enrichment media) which favor the growth of the typhoid bacillus (and other intestinal organisms) and inhibit other bacteria. Then by means of plate cultures the typhoid and the colon bacilli have been differentiated. Isolation of a specific pathogen, however, is but rarely attempted and is of scientific rather than practical interest.

The sewage bacteria for which search is usually made are *Escherichia coli* (and closely related *Aerobacter aerogenes*) and streptococci. In England certain spore-bearing anaerobes of the genus *Clostridium* are sometimes sought. The methods used may be divided into two general groups, the presumptive methods and those in which definite identification of *Escherichia coli* is made.

Presumptive Methods. Advantage is taken of certain of the physiological characters of *E. coli* to differentiate it from other organisms. The media commonly used are lactose broth and lactose bile in fermentation tubes. When various amounts of water to be examined are inoculated into fermentation tubes containing lactose broth kept at blood heat for twenty-four hours, gas will be produced in those tubes in which *E. coli* is present. It seems safe to infer that *E. coli* is not present if the gas is not produced in any of the tubes inoculated. The reverse, however, that *E. coli* must necessarily be present whenever gas is produced, does not hold good, for there are other organisms which can ferment lactose with the production of acid and gas. The test is more definite when a 2 per cent solution of *lactose bile* is substituted for the lactose broth in tubes. The bile inhibits the growth of most organisms not of intestinal origin. Gas production, under these conditions, therefore, in a large proportion of cases means the presence of *E. coli*. The absence of gas production indicates that this organism is not present. These tests are termed *presumptive tests*, because while they enable one to recognize waters which are suspicious, they do not certainly identify the organism responsible for the gas in the suspicious cases. This permits one, in other words, to divide waters tested into two groups, those which are certainly good and those which are suspect. The latter require further examination.

Examination of Water and Sewage—Standard Methods. There are no accepted international standard methods. In the United States it is customary to follow "Standard Methods for the Examination of

Water and Sewage" as prepared, approved, and published jointly by the American Public Health Association and the American Water Works Association. We are here concerned primarily with the fourth section of Standard Methods having to do with the bacteriological examination of water and with tests to determine the degree of purification of sewages.

As has been noted previously, assessment of the potability of water is usually based upon determination of the approximate number of organisms of intestinal origin related to *Escherichia coli*. This group of organisms has been given several names; perhaps most commonly the *coliform* group. This is defined as including all aerobic and facultative anaerobic gram-negative non-spore-forming rod-shaped bacteria (bacilli) which produce gas in lactose broth.

Three types of tests may be carried through to determine the occurrence of coliform bacteria in water; the *presumptive test*, the *confirmed test*, and the *completed test*.

The *presumptive* test is made by inoculation of varying dilutions of the water being assayed into a series of fermentation tubes of lactose broth or lauryl sulfate lactose broth. In cases in which detection of coliform organisms in a considerable amount of water, such as fifty- or hundred-milliliter quantities, is attempted large tubes or bottles must be used in which have been placed amounts of double or quadruple strength lactose broth so that upon dilution by the addition of water the broth concentration will be standard. The production of gas within approximately twenty-four hours at 35°–37° C. is *presumptive* evidence that coliform organisms were present in the inoculum of the tubes showing gas. If gas is present in forty-eight hours but not in twenty-four hours, the result is classified as *doubtful*, and a confirmed or completed test is carried through. If no gas is produced, it is assumed that no coliform bacteria were present in the inoculum.

All cases of doubtful reaction, and preferably positive presumptive reactions as well, should be tested further by use of a *confirmation* test. A confirmation may be made by transfer of a loop of the broth to any one or more of several media. It may be streaked on the surface of Endo agar or on eosin methylene blue agar in plates, or inoculated into a tube of brilliant green lactose bile. If colonies typical of *Escherichia coli* are produced on the agar or if gas is produced in the brilliant green lactose bile, the presence of coliform organisms is confirmed.

If atypical colonies only are produced, transfers of such colonies should be made and pure cultures grown on agar slant and in lactose broth. If the organism belongs to the coliform group, stained preparations will show it to be rod-shaped, gram-negative and without spores, and gas should be produced in lactose broth.

The student in carrying out a test should consult and follow in detail the directions found in Standard Methods.

A discussion of the cultural and physiological characteristics of the several species of coliform bacteria will be deferred to Chapter 34, in which the health significance of the entire group of intestinal bacteria (enterobacteria) will be considered.

Identification of Escherichia coli. Many methods have been devised for the differentiation of the colon bacillus either directly from water or from the lactose broth or lactose bile tubes discussed above. When water containing *E. coli* is plated on agar containing 1 per cent lactose and sufficient litmus solution to color it blue, and is then incubated for twenty-four hours at blood heat, the lactose-fermenting colonies will be found to be surrounded by a red zone.

More frequently use is made of Endo agar or eosin methylene blue agar in confirming the results of positive presumptive fermentation tests noted above. Endo medium is lactose nutrient agar containing the dye fuchsin which has been decolorized by the addition of sodium sulfite. A loopful of broth from a fermentation tube showing gas production is spread over the surface of the medium. *E. coli* is characterized by the restoration of the red color in the medium about its colonies and the development of a green sheen on their surfaces. The eosin methylene blue medium is also a lactose nutrient agar containing a suitable concentration of these dyes. *E. coli* grows well, and develops a dark colony with a green sheen; *Aerobacter aerogenes* likewise develops well with the formation of colonies without the metallic sheen. These media are used extensively, as the finding of *A. aerogenes* may not have the same sanitary significance as the presence of *E. coli*.

Interpretation of Results. It is probable that *E. coli* may actually gain entrance to water in small numbers without necessarily indicating serious sewage contamination. Water wholly free from *E. coli* is certainly above suspicion. A standard commonly accepted is that water containing less than one viable cell of *E. coli* per fifty cubic centimeters is safe for use, while water containing more than that number is probably contaminated with sewage and should not be used. It should be emphasized that the reason for avoiding water containing *E. coli* is not that this organism itself is necessarily harmful when taken into the body, but because of the fact that it indicates sewage pollution and the possible presence in such polluted water *sooner or later* of pathogenic microorganisms from the intestines.

Purification of Water

Water at rest in ponds or lakes or running in streams tends to purify itself. This is due to a variety of causes. The first factor is *dilution;* sewage entering a stream becomes greatly diluted. The organisms present in impure water tend to settle to the bottom, or undergo *sedimentation.* Bacteria tend to clump together or to attach themselves to the surface of solid particles in the water and are carried to the bottom with these particles. Once at the bottom, conditions are usually so unfavorable for continued growth of many species that death soon occurs. This is particularly true with respect to pathogenic forms that may be present. The water of any flowing stream also usually undergoes a certain amount of *filtration.* A portion of the water flows through the sand in the bed of the stream; algae and other water plants tend to retard the current and to catch microorganisms which may be present. It is probable that *light* may sometimes destroy the bacteria in water, but its effect is not felt at any distance from the surface, and in most cases it is not of primary importance. Microorganisms characteristic of sewage, particularly the pathogenic forms, generally find the temperature and food conditions in water not suitable for continued growth. This is due in part to the usually rapid removal of organic matter resulting from the activity of bacteria oxidizing such material. In addition, when water is highly polluted, the putrefactive and decay-producing bacteria develop so rapidly that the pathogenic forms are destroyed as a result of *antibiosis. Bacteriophages* of many kinds and specificities may be isolated from sewage and may be significant in destruction of some microorganisms. Many species of *protozoa* live in polluted water, their food consisting of bacteria and related microorganisms. These protozoa are often of major significance in reducing the numbers of bacteria.

Disposal of Sewage. Sewage is water containing a considerable proportion of fecal material and of household and manufacturing wastes of different kinds. Care should be used in its disposition for two principal reasons: first, because when allowed to stand in pools it may develop odors and create a nuisance; second, it may pollute water supplies, make dangerous the use of natural waters for recreational purposes, and kill fish. Many methods of disposal of sewage have been advocated, most of them successful under certain conditions, but none of them seemingly applicable to all conditions. The easiest method of sewage disposal and one which is quite generally used is to allow it to flow into a stream, lake, or some other body of water. If the stream is of sufficient size so that there is not such a concentration of sewage as to create a nuisance and the water is not used for domestic or recreational purposes it has been claimed that there should be no serious objection to this method of disposal. The same is true with reference to sewage allowed to run into the ocean or some arm of the ocean where it is continually washed away by the action of the tide and greatly diluted, or where the sewage empties into a lake. In some cases, however, pollution seriously limits the recreational value of a stream or lake, and should therefore be eliminated. There are many cases where cities use a lake both for the disposal of sewage and as a source of drinking water, and other instances where one city pours its sewage into a river used by another city as a water supply. In such cases other methods of sewage purification and disposal should be sought. The disposal of sewage of inland towns which are not situated on the banks of streams of considerable size is attended with difficulty. Many of the towns of the Middle and Western states of the United States, for example, dispose of their sewage by allowing it to run into some water channel which during a portion of the year has a flow of water quite insufficient for adequate dilution. Sewage under these conditions becomes stagnant and creates a nuisance as a result of development of unpleasant odors.

The solid material in suspension and part of that in solution may be removed from sewage by the use of coagulants. This method of disposal has been extensively used in England. The sewage is allowed to run into large vats where alum, lime, sulfate of iron, or some similar compound is added and thoroughly mixed. This causes a flocculation and sedimentation of the solid material. The clear sewage is then allowed to run into a stream. A sufficient quantity of organic matter is usually removed to insure the absence of putrefaction and of the development of obnoxious odors. The precipitated material or sludge may be burned or utilized as fertilizer.

Some of the large cities of Europe, such as Berlin and Paris, and some of the cities on the western coast of the United States dispose of their sewage by means of irrigation sewage farms. This can be economically carried out where large areas of sandy land are available and where the climate is dry. With a heavy soil and moist climate, however, it is not very practicable. The sewage is spread out over the land as is customary in irrigation, and the excess is carried off by a system of underground tiles. Crops are grown upon these sewage farms and are a source of considerable income to those cities where this system is used. The organic material of the sewage is quite completely oxidized by passage through the soil, and the water leaving by the under drain is of at least as high a quality as that of the streams into which it flows. It is not considered advisable to raise vegetables for human consumption upon such land. Grain crops and hay are generally grown.

The fact that under appropriate conditions the bacteria of sewage can themselves effect a very considerable purification has led to the development of various structures and devices for encouraging their growth. Such, for example, are various types of septic tanks, contact beds, filters, and activated sludge chambers used for sewage treatment. A *septic tank* is a closed chamber or cistern into which sewage is allowed to flow. It is of sufficient size so that from twelve to twenty-four hours are required for any given portion to pass through. The solid particles settle to the bottom of the tank. Aerobic bacteria quickly utilize all the free oxygen that may be dissolved in the sewage when it reaches the septic tank. Under these conditions certain of the anaerobic and facultative putrefactive bacteria bring about rapid solution of the solid organic materials. If the sewage is allowed to remain in the tank for too long a period, this decomposition goes on to the production of malodorous compounds, but when the flow is properly regulated, the action upon the constituents of the sewage within the tank is essentially digestive. The cellulose of paper, other carbohydrates, proteins, etc., are thus converted into soluble compounds and gases. In some cases the effluent is allowed to empty directly into a stream, but in most cases further purification is advisable. This is accomplished by means of some form of apparatus that will allow for rapid oxidation. The type first used was the *sand filter*. A filter bed is constructed by covering drain tile with crushed rock, gravel, and sand. The sewage from the septic tank is allowed to pass into a smaller chamber, termed a dosing chamber, which fills gradually and then empties quickly by means of automatic siphons out upon the surface of this sand filter. It has been found in practice that the sand grains become coated with a layer of bacteria, usually more or less gelatinous. Air penetrates the sand, particularly between the dosing periods, so that the bacteria present under these aerobic conditions rapidly oxidize practically all the organic material still remaining in the sewage. The action may be compared to that which occurs in the quick vinegar process, where an alcoholic solution is allowed to trickle into the top of a cask over beech shavings covered with acetic acid bacteria and reappears transformed to vinegar at the bottom of the cask. The organic materials are adsorbed by these organisms coating the sand grains. The nitrogenous compounds are converted first into nitrites and then into nitrates. Sulfur appears in the effluent of the bed in combination as sulfates. Carbon is converted into carbon dioxide, and hydrogen to water. The effluent from a sand filter which is working properly contains relatively few bacteria and little dissolved organic matter. Raw sewage as it enters the septic tank contains a considerable percentage of organic matter and from several hundred thousand to as many million bacteria per milliliter. The bacteria do not decrease materially in numbers during passage of the sewage through the septic tank, though many species which enter are quickly destroyed, while those for whom the environment is favorable multiply rapidly. The tank flora quickly becomes stabilized. In passage through the filter beds the numbers of bacteria are very greatly decreased; sometimes in an efficient filter bed they will be below one hundred per cubic centimeter, the percentage of bacterial elimination thus being considerably higher than 99 per cent. At the same time the organic material is almost completely oxidized. Sewage treated in this manner can be passed into a stream without fear of creating a nuisance because of the development of odors or of endangering health.

Contact beds are modifications of sand filters. They consist of chambers or beds filled with crushed rock, slate, cinders, or coke. The sewage from the dosing chamber is allowed to run rapidly into one of these beds until it is entirely filled. It remains in contact with the material in the bed for a short time and is automatically drawn off. Air follows the sewage down and comes in contact with the surfaces of all portions of the bed. The sewage as it passes through the bed is thoroughly aerated. The surfaces of the materials constituting the bed become heavily encrusted with a layer of oxidative bacteria and protozoa which feed upon microorganisms. In this film the organic matter of the sewage is adsorbed and rapidly oxidized. Most of the bacteria originally entering also adhere and are for the most part destroyed. Oxidation proceeds rapidly, and the sewage when emptied into a stream usually sufficiently aerated so that it will not cause a nuisance.

A trickling filter is essentially a modification of a contact bed. It consists of a bed or vat filled with crushed stone or cinders on which the sewage from a septic tank is spread by means of a sprinkling device. The liquid passes down over the surface of the crushed rock, etc., and the dissolved organic matter is oxidized in the same manner as has been described. The drain of the bed is left constantly open so that it never fills with sewage. The oxidation in such a properly constructed bed is extremely rapid, and the effluent will prove stable and inoffensive when allowed to flow into a stream.

Sewage, more particularly the effluent of septic tanks, is sometimes purified by the addition of disinfectants. Those most commonly used are chlorine and the hypochlorites.

Whether or not sewage has been sufficiently purified so that it may properly be discharged into a stream or a body of water is determined not only by the danger from pathogenic bacteria but also by the amount of undecomposed and readily decomposable organic matter it may contain. If there are considerable quantities of such organic matter, the oxygen dissolved in the water of the stream or other body of water may not be sufficient to bring about complete oxidation. Under such conditions the oxygen of the stream is depleted to a point where conditions may be essentially anaerobic and fish are killed. Such depletion is particularly likely to happen in winter when ice covers the stream and aeration of the water below the ice is very slow and inadequate. In extreme cases, the concentration of organic matter may be sufficient to lead to putrefactive anaerobic changes with development of malodorous gases such as hydrogen sulfide. Methods have been devised to determine the amount of oxygen needed to complete the oxidation of the organic matter present and to tell whether its addition to a stream will prove destructive to aquatic life. The method most commonly used to test sewage is by determination of the *biochemical* (or *biological*) *oxygen demand.*

The biochemical oxygen demand (abbreviated as B.O.D.) is usually evaluated by the rate of disappearance of oxygen in water to which has been added in varying amounts the sewage or sewage effluent being tested. The B.O.D. is defined as the parts per million of oxygen required to bring about stabilization of the decomposable organic matter through the agency of aerobic microorganisms. The bottles used for incubation of the sample should have a capacity of two hundred fifty to three hundred milliliters, be carefully cleaned, and be fitted with ground glass stoppers. A standard dilution water is prepared by adding to distilled water small quantities of solutions of ferric chloride, calcium chloride, magnesium sulfate, and a phosphate buffer containing potassium acid phosphate brought to a pH of 7.2 and with the addition of ammonium sulfate. Various dilutions of the water of sewage to be tested are made in standard dilution water, placed in the bottles, tightly sealed without air, and incubated for five days at 20° C. The various dilutions are examined by standard techniques to determine the amount of dissolved oxygen remaining. From the results one may calculate the demand upon a stream or other body of water for oxygen to oxidize the organic matter, and whether there has been sufficient treatment of the sewage to avoid too serious depletion of the oxygen with its consequent deleterious effect upon aquatic life.

Another method of getting at the relative stability of sewage effluents is to fill a glass-stoppered hundred and fifty milliliter bottle with the water to be examined, carefully add below the surface 0.4 milliliter of 0.05 per cent methylene blue. The bottle is incubated at 20° C. until decolorization has taken place. The results are reported either in terms of the number of days required for the decolorization of the methylene blue or by use of so-called relative stability numbers taken from a table prepared for easy determination of the relative stability by use of the equation

$$S = 100 - (1 - 0.794^t)$$

in which S is the stability in per cent and t is the number of days required for the decolorization of the methylene blue. For example, if decolorization occurs in one-half day, the value of S is 11, in five days, 68, and in twenty days, 99.

Purification of Water for Domestic Use. Water from deep driven wells is usually free from sewage contamination. This may not be true, however, if the stratum from which the water is drawn is cavernous limestone, for in many cases there may be direct connection between the ground water and the surface through "sinks" into which streams or run-off water may empty. Large cities are rarely located under conditions that make a ground water supply possible. Most of these large cities secure their water supply from lakes, reservoirs, or streams. Some cities have solved the problem by using water which has been gathered on an uninhabited and protected watershed. Many of our western cities, for example, secure water from mountain lakes or streams. New York City has created a great system of reservoirs and is carefully protecting them from pollution. Chicago secures its water supply from Lake Michigan.

Many cities partially purify their water by holding it for a time in large reservoirs before use. The factors already discussed under the natural purification of water, such as sedimentation and antibiosis, are active, and if sufficient time is allowed, these efficiently dispose of most of the organisms present. This method is frequently rendered more efficient by the use of coagulants which carry down in the coagulum all silt and most of the bacteria which are present.

Impure water supplies are in some cases more or less completely sterilized by the use of disinfectants. The most important of these are chlorine, the hypochlorites, and ozone. It has been found that the addition of chlorine or calcium hypochlorite to water quite effectually destroys all bacteria which are present while the free chlorine rapidly disappears, and the water does not seem to be seriously injured for domestic purposes by the time it passes through the distributing pipes. Water is purified for use in a few European cities by being exposed in thin sheets to a current of air which has been heavily charged with ozone developed by electricity.

In recent years some use has been made of ultraviolet light for the purification of contaminated water. The Cooper-Hewitt mercury vapor lamp gives off a very large proportion of these rays. If water is allowed to pass in a thin sheet over such a lamp, all pathogenic bacteria are rapidly destroyed.

Another common method of purification of water by cities is by the use of sand filters. These consist of layers of sand, usually a foot or more in thickness, through which the water is allowed to pass by gravity and collect in underground drains. It is then pumped to the city mains. These filters are not very efficient when first constructed, usually several days or a week elapsing before the maximum decrease in the number of bacteria in the filtered water is obtained. A filter of this kind owes its efficiency, in part at least, to the development on the surface of the sand grains of certain organisms which form a more or less gelatinous film and prevent the passage of pathogenic organisms and eventually destroy them. The upper layer of such a filter must occasionally be removed on account of clogging, and a new layer of sand must be substituted. The water from such a filter should not be used for a few days after this replacement in order to give the bed an opportunity to reach its maximum efficiency. The usefulness of properly constructed filter beds of this type has frequently been demonstrated by the reduction of the death rate in cities where such a system is used.

Filters constructed of unglazed porcelain are occasionally used in the home and in the laboratory for filtering impure water. Some of these are constructed to fit directly upon the tap. Such filters, if properly made, are efficient in the beginning, the water coming from them being sterile, but after a time bacteria grow through the pores of these filters and are then to be found in considerable numbers in the water which passes through, frequently in greater numbers than are present in the original supply. It is evident, therefore, that such filters if used at all must be thoroughly cleaned and heated to red heat at short intervals if they are to retain their efficiency.

When it is impracticable to purify water for domestic use by any of the means described above, all pathogenic microorganisms may be destroyed by boiling. The temperature of boiling water is sufficient to kill any of the bacteria pathogenic for man which gain entrance to the body through the alimentary tract.

CHAPTER 26

Microorganisms in Soils

In chapter 20 there were discussed the cycles of several of the elements in nature and the overall relationships of microorganisms to these cycles. Each of these consists either of alternate building up into organic compounds and the disintegration of these into inorganic compounds or of alternate oxidations and reductions. These chemical changes are in many cases of considerable economic significance, particularly in soil, and are of great importance in agriculture from the standpoint of crop production and soil fertility. Some are also important in the purification of sewage and trade wastes; an understanding of them is essential to an insight into such phenomena as the self-purification of streams. In some cases the changes have been of some geological significance.

TRANSFORMATIONS OF NITROGEN AND ITS COMPOUNDS BY MICROORGANISMS

Microorganisms capable of bringing about changes in nitrogenous compounds may be divided into four principal groups: first, those which are capable of fixing atmospheric nitrogen, that is, of combining free nitrogen with other elements or compounds (these new compounds are utilized by the organism in building up organic compounds which may be used in cell metabolism); second, those which oxidize ammonia and nitrites; third, those capable of reducing nitrates and nitrites, and fourth, those which bring about changes such as ammonification in organic nitrogenous compounds.

Fixation of Free Nitrogen. *Assimilation of Free Nitrogen.* In the discussion of the nitrogen cycle it was noted that certain groups of microorganisms have the ability to fix or assimilate atmospheric nitrogen. Among these are a few bacteria and probably some molds and algae. These organisms are of very real importance from the standpoint of agriculture. The nitrogen of the air is in general quite unavailable as a nutrient to plants and animals. Animals use only the nitrogen compounds, largely proteins, secured directly or indirectly from plants. Most plants require that nitrogen, to be assimilated, be in the form of ammonia or of nitrates. The amount of nitrogen in a soil in the form of ammonia or of nitrates is an important factor in its fertility; an increase usually increases crop yields. Any method which will bring the nitrogen from the air into combination so that it will eventually add to the soil nitrogen is advantageous.

Several commercial methods of fixing atmospheric nitrogen have been developed for the manufacture of nitrogenous agricultural fertilizers. The great importance of nitric acid and nitrates in the manufacture of munitions during both World War I and World War II stimulated the synthetic production of nitrates, as natural supplies, such as from Chilean nitrate, were quite inadequate. Technical advancement has been so rapid that a considerable part of the nitrogen now used in commercial fertilizers is synthetic in origin, in the form of ammonia, nitrate, or urea. The consumption of synthetic nitrogen compounds has greatly increased. However, it is well to emphasize that far more nitrogen is fixed and made available to agriculture through the activity of microorganisms than through chemical synthesis.

Gaseous (atmospheric) nitrogen is relatively inert. It combines with other elements as hydrogen or oxygen with some difficulty. In commercial fixation it has been found always necessary to use a catalyst to expedite the reaction. In one of these commercial processes (the Haber process) the reaction of a mixture of nitrogen and hydrogen under the right physical conditions is catalyzed by iron with formation of ammonia. Essentially the reaction is

$$N_2 + 3H_2 \rightarrow 2NH_3$$

Certain kinds of bacteria when growing in a medium which contains a suitable carbon food and nitrogen only in the form of the free gas are able to build up protoplasm, which contains, of course, a considerable percentage of nitrogen in combination. Inasmuch as most microorganisms are able to synthesize their cell proteins from suitable carbon compounds and ammonia, it is assumed that these organisms catalyze a change which has been termed a "biological Haber process." Positive proof that the product of the catalysis is ammonia is lacking; it is possible that intermediate compounds such as hydroxylamine are produced. However, the presence of an enzyme catalyst, *nitrogenase,* has frequently been postulated. Careful study has added much to our knowledge of the conditions under which microorganisms may fix nitrogen, and various possible steps have been proposed to explain the process, but adequate proof of the validity of any of the suggestions is not yet at hand. The microorganisms which fix atmospheric nitrogen may conveniently be placed in two groups: those which grow symbiotically with certain other plants, and those which live free in soil or water.

Symbiotic Nitrogen-Fixing Organisms. Two types of organisms are known to live in symbiotic relationship to higher plants, certain fungi and certain bacteria.

As previously noted, many higher plants have fungi which grow on the roots, in some cases internally and in others externally. These combinations of roots and fungous hyphae are termed *mycorrhizas.* Fungi are quite commonly present upon the roots of certain trees, particularly when grown in a somewhat sterile and acid soil. The roots of pines, oaks, beeches, etc., may harbor such organisms. Many of the plants which are native to the acid soils of peat bogs, such as the heathers and certain of the orchids, grow symbiotically with a mold. The roots of the alder, the Russian olive, and the shrub Labrador tea of the midwest prairies generally produce nodules or tubercles of considerable size in which some microorganisms, a mold or bacterium, develop. Some investigators have believed that mycorrhizas are important in taking up nitrogen from the air and in converting it into a form so that it is ultimately utilized by the plant upon which the organism is growing. Quite certainly in many forms the symbiotic fungus has nothing directly to do with the nitrogen metabolism of the plant; however two genera of bacteria (*Rhizobium* and *Mycobacterium*) are known definitely to include species which grow in symbiosis with higher plants.

Members of the genus *Rhizobium* occur upon the roots of most leguminous plants, such as pea, bean, vetch, alfalfa, sweet clover, soybean, and clover. The bacteria enter the roots through the young root hairs. The root cells respond by multiplication and growth with the consequent development of a nodule or tubercle in which the organisms find favorable conditions for rapid growth and development. Carbon compounds are supplied to the organism by the plant. In some unknown manner the bacteria and root cells in symbiosis assimilate atmospheric nitrogen, a part of which ultimately becomes available to the legume as food. The ability on the part of leguminous plants to take up nitrogen and incorporate it into their bodies in this manner, through the agency of the organisms on the roots, is most important in agricultural economy. Leguminous crops when grown and plowed under add appreciably to the fertility of the soil because of the very considerable contribution of nitrogen which they make. Legumes are necessary in crop rotations designed to maintain or build up soil fertility.

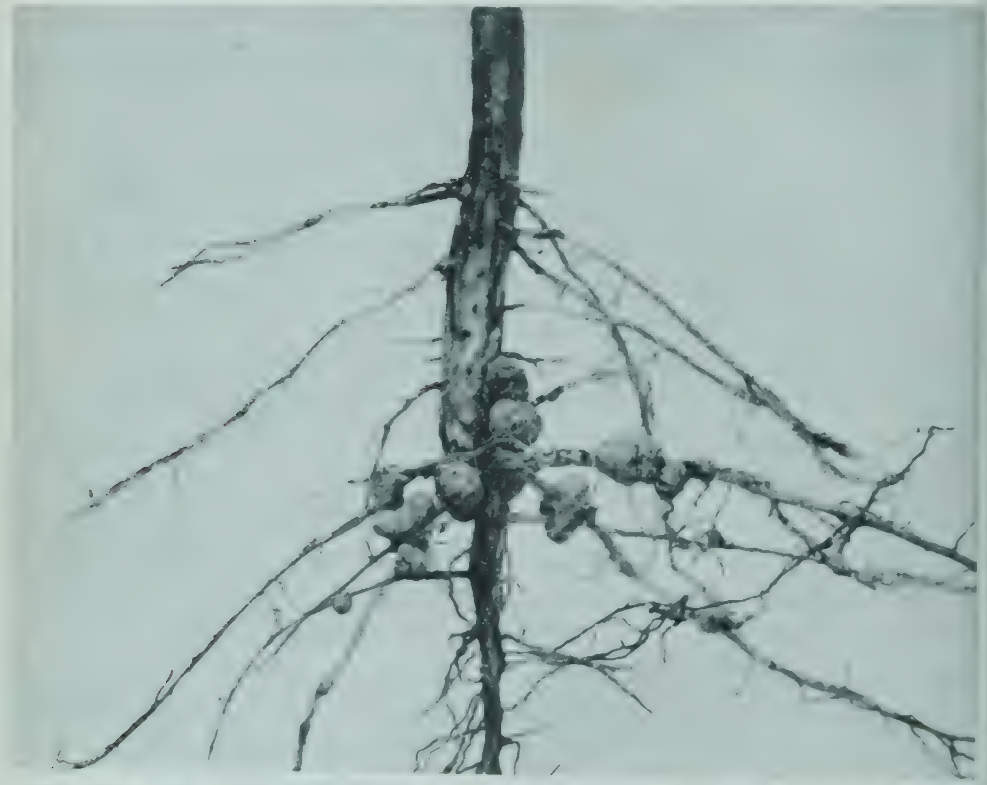

Fig. 26–1. Root nodules on the roots of the soybean. These nodules contain bacteria of the species *Rhizobium japonicum* which in symbiosis with the plant are able to fix atmospheric nitrogen and make it available to the soybean.

The nodule bacteria may be grown readily on suitable media in the laboratory. However, it has thus far proved impossible to find conditions in media suitable for the demonstration of any nitrogen fixation. This occurs apparently only when the organism is growing symbiotically in the root nodule.

The nodules of leguminous plants have been found commonly to contain a red pigment which resembles closely the hemoglobin of the blood. It has been termed both hemoglobin and leghemoglobin.[1] Virtanen (1947) claims that only nodules of leguminous plants that show this red pigment are able actively to fix atmospheric nitrogen. He also contends that the bacterial strains most effective in inducing N fixation in a nodule are those that are associated with higher content of the pigment. Although proof is not adequate as to the exact function performed by this hemoglobin, the evidence seems to favor the concept that it is in some way related to nitrogen fixation. The young cells of the *Rhizobium* are rod-shaped and actively motile by means of one to several flagella, and gram-negative. When, in the soil, an organism comes in contact with a root hair of a suitable leguminous plant, the root hair is stimulated to increased growth, the bacterial

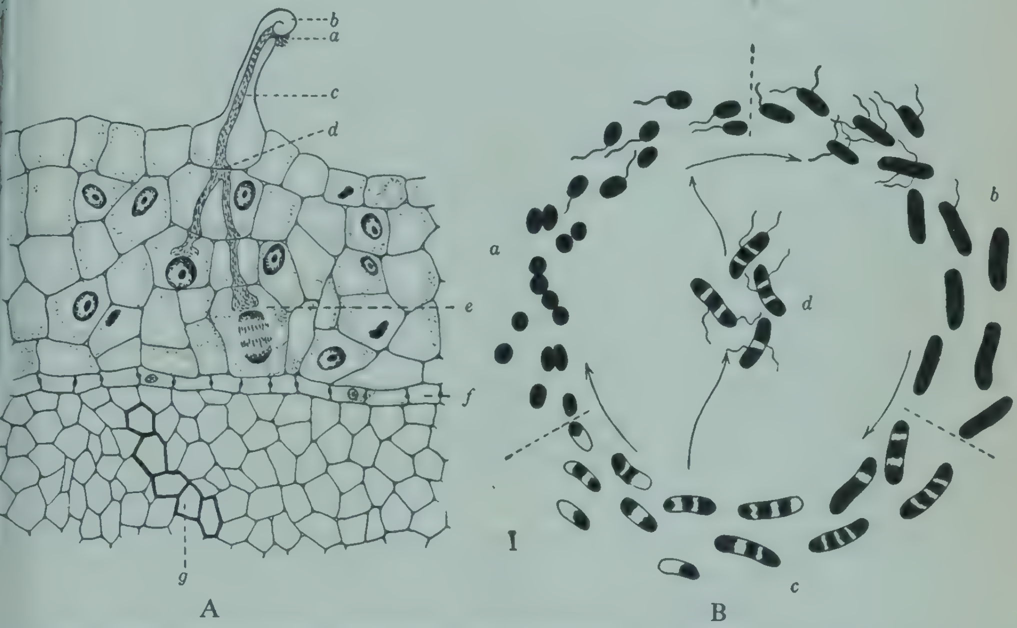

Fig. 26–2. A. Invasion of root by nodule bacteria. *a*, bacteria in soil; *b*, coiled tip of root hair; *c*, "infection thread" of bacteria; *d*, enlargement of "infection thread" at cell wall; *e*, root cell showing cell division and beginning of nodule formation; *f*, endodermis of root. (Adapted from Thornton.) B. Thornton's scheme of the life cycle of *Rhizobium*. *a*, Coccoid cells, becoming motile, then elongating (*b*) and becoming bacteroids (*c*).

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[1] *leg-* from *legume*.

cell penetrates to the interior of the hair and begins to multiply. A mass of bacteria developing as a filament grows to the base of the hair and penetrates through the cell walls into some of the root cells. The neighboring cells are then stimulated to growth and multiplication, and a nodule develops. The shape and size of the nodule varies with different species of legumes, but in all cases certain areas develop large cells which become filled with bacteria. When growing in the cells of the root nodule, the bacteria frequently assume unusual shapes: sometimes they become swollen, in other cases they branch. These bizarre shapes were observed by some of the earlier workers before they were known positively to be bacteria. Because of their resemblance to bacteria they were termed *bacteroids;* this name is still sometimes used.

Even the earlier workers observed differences among the organisms producing nodules on different species of leguminous plants. It was assumed, however, that they constituted different varieties of a single species which was named *Rhizobium leguminosarum*. Usually one species of *Rhizobium* will cause nodule formation only on certain species of legumes, not on others. For example, the organism from the nodules of the alfalfa will also produce nodules on the sweet clover, but not on the soybean. There have been thus discovered a number of cross-inoculation groups among the leguminous plants. The organisms characteristic of these groups may be regarded as species. They are named usually after one of their host plants. Six species are now usually recognized: *Rhizobium leguminosarum* which is found in the nodules on pea, lentil, vetch, and sweet pea; *R. phaseoli* from the true beans; *R. trifolii* from the true clovers; *R. lupini* from lupine, serradella, and *Ornithopus; R. japonicum* from soybeans; and *R. meliloti* from sweet clover, alfalfa, the medics, and *Trigonella*.

Leguminous plants are very widely distributed in nature. Species of legumes of one kind or another are found from the tropics to the polar regions, from sea level to above the timber line on the mountains, in moist soils and dry soils, in acid soils, neutral soils, and alkaline soils. The plant family, *Leguminosae*, in other words, is cosmopolitan. Yet wherever these plants are found, examination will usually reveal the nodules and the enclosed bacteria. Because of their distribution and abundance, growth of native legumes has had much to do with initial building up of the fertility of virgin soils. Furthermore, other plants such as grains or grasses when grown with a legume, as clover, may show some direct benefit. It is believed that some of the nitrogen fixed by the legume may diffuse into the soil. The presence of clover or other legumes in meadows and pastures leads to more luxuriant growth and greater persistence of the grasses.

In some cases the kind of bacterium needed for nodule formation of a particular legume may not be present in a soil or the strains present are inefficient in symbiotic fixation in the nodule. The plant may grow nevertheless, provided there is a plentiful supply of nitrogen already in the soil, otherwise as soon as the plantlet has exhausted the nitrogenous reserve food of the seed, it will turn yellow and eventually will die. It has been found profitable to inoculate seed or soil with the appropriate organisms when they are needed. This is frequently done for such agricultural crops as alfalfa and red clover. The field may be inoculated by spreading soil containing the bacteria, or, more commonly, by inoculating the seed by means of pure cultures grown in the laboratory. These may be obtained from commercial houses.

Many species of plants belonging to the tropical plant family *Rubiaceae* are known to harbor symbiotic bacteria in nodules in their leaves. The organism present is an acid-fast, non-motile, non-spore-bearing rod. It has been named *Mycobacterium rubiacearum*. It is claimed that this organism serves much the same function as do the legume bacteria in fixing nitrogen. The evidence is not as complete, however.

Non-Symbiotic Nitrogen-Fixing Bacteria. The free-living nitrogen-fixing bacteria are of two types, anaerobic and aerobic.

The anaerobic nitrogen-fixing bacteria most studied belong to the genus *Clostridium*. *Clostridium pasteurianum,* one of the commoner forms, is a member of the group of butyric acid bacteria and has been found to be one of the more efficient of the nitrogen-fixers, when studied in the laboratory, but it is not entirely clear that conditions are often right in the soil for its activity.

Many other soil clostridia are able to fix nitrogen when supplied with suitable carbon nutrients. In fact Rosenblum and Wilson have shown that of fifteen different species of the genus studied only three consistently failed to fix atmospheric nitrogen.

Kamen and Gest (1949) have shown that an organism of a quite different group of bacteria has also some powers of nitrogen fixation under anaerobic condition. *Rhodospirillum rubrum,* one of the purple bacteria, was found to produce hydrogen gas in a special medium containing malic acid when illuminated and to be able to fix free nitrogen.

This is of interest because it has shown the occurrence of nitrogen fixation in a relatively simple synthetic medium and has increased the possibility of discovering the intermediate steps in fixation.

The genus *Azotobacter* includes most of the aerobic free-living bacteria which have been definitely shown to assimilate atmospheric nitrogen.

Several species have been described (as *Azotobacter chroococcum*, *A. agilis*, and *A. indicus*). They are rather plump, rod-shaped bacteria, non-spore-forming, often motile. They grow readily on laboratory media containing carbohydrates or related alcohols (particularly mannitol) and utilize atmospheric nitrogen. Wilson (1944) has shown that when ammonia is present in the medium, *Azotobacter* uses this and does not fix atmospheric nitrogen. *Azotobacter* is quite abundant in many fertile soils and probably in a measure is responsible for the accumulation of nitrogen in the soil. Certain soil-inhabiting blue-green algae such as *Nostoc muscorum* also fix atmospheric nitrogen, apparently by the same mechanism as does *Azotobacter*.

It should be understood that most, if not all, of the nitrogen fixed by these free-living soil forms is not at once freed and made available to higher plants. For the most part, at least, it is built into protoplasm and only becomes available upon the death and disintegration of the bacterial cells.

Oxidation of Ammonia and Nitrites. In the discussion of the nitrogen cycle it was noted that the ultimate nitrogenous decomposition product of proteins is in all cases ammonia. This is afterwards converted into nitrites and then into nitrates by certain species of bacteria. This fact is of considerable importance from two points of view, first, that of agricultural practice, and second, in the recognition of pollution of water and water supplies.

Most green plants utilize either nitrates or ammonia as sources of nitrogen in the soil. Ammonia produced by the decomposition of manures and organic matter may not be directly available even to those plants which can use it, for it is readily leached from soil and is usually converted to nitrate as rapidly as it is formed. This change to nitrates in the soil is brought about by two or three species of bacteria, belonging to the genera *Nitrosomonas* and *Nitrosococcus*. They do not grow on the usual organic laboratory media, and they are autotrophic, using carbon dioxide as a carbon source. The overall reaction may be represented as follows:

$$2NH_3 + 3O_2 \rightarrow 2HNO_2 + 2H_2O$$

The steps or intermediate products are not known. The transformation is sometimes termed *nitrosification*. Nitrites are even less available to green plants than is ammonia and, when they accumulate in any considerable quantity, are distinctly poisonous. They are, however, ordinarily converted at once in the soil into nitrates by the action of species of the genus *Nitrobacter*. The reaction may be represented by the following equation:

$$2HNO_2 + O_2 \rightarrow 2HNO_3$$

This transformation is termed *nitrification*. The nitrous and nitric acids do not usually exist uncombined in the soil, but as nitrite and nitrate. The latter may then be taken up by green plants.

Under some conditions nitrates may be leached from one soil and concentrated in another. The Chilean nitrate so important as a nitrogen source for commercial fertilizers has apparently thus been sufficiently concentrated so that it may be extracted profitably. Occasionally so-called nitre spots may occur in prairie or semiarid soils on which nitrates are so abundant as to interfere with normal plant growth. The same changes, which have been discussed as occurring in soil, occur also in water which has become contaminated with organic matter, such as sewage. The presence of ammonia in water is generally held to indicate a comparatively recent contamination with some organic matter. When the nitrogen is present in the form of nitrites, it is understood that oxidation has been partially effected or that nitrates have been partially reduced by fresh contamination. The presence of nitrogen solely as nitrate in water indicates that at some time in the past the water has probably been contaminated with organic matter, but that complete oxidation has occurred. By studying the relative proportion of ammonia, nitrites, and nitrates in a sample of water the chemist is frequently much aided in judging its potability.

Reduction of Nitrogenous Compounds. In the presence of an abundance of organic matter and in the absence of free oxygen, certain bacteria are capable of reducing nitrates to nitrites, and nitrites to free nitrogen gas. Under anaerobic conditions the nitrate serves as a hydrogen acceptor in the oxidation of organic matter. This process is termed *denitrification*. It may occur to a certain extent in foods such as hams which are preserved by the use of saltpeter ($NaNO_3$), the nitrite formed combining with hemoglobin present to form a bright red pigment. Denitrification may also occur in soils which are waterlogged. It has been found, for example, that the addition of nitrates to a soil kept flooded for the growing of a crop such as rice may be detrimental. Because of the anaerobic condition established in such a soil, the nitrates are converted into growth-inhibiting nitrites. It is found that crops as rice grown in mud take up their nitrogen in the form of ammonia and related compounds rather than as nitrates.

The bacteria which are able anaerobically to reduce nitrates are quite common. They include species belonging to many genera. Bacteria able to reduce nitrates with production of free nitrogen belong for the most part to the genus *Pseudomonas*. One interesting member of the genus *Thiobacillus* is able to grow under anaerobic conditions in the presence of free sulfur and nitrate. It reduces the latter, producing free nitrogen and simultaneously oxidizing the sulfur.

TRANSFORMATION OF SULFUR AND ITS COMPOUNDS

As with nitrogen, so with sulfur one finds both oxidation and reduction brought about by the activity of soil microorganisms.

Oxidation of Sulfur and Its Compounds. Hydrogen sulfide is oxidized by certain water bacteria such as species of *Beggiatoa* and of soil bacteria such as *Thiobacillus* with the production of free sulfur and sulfuric acid. Organisms of this type are very common in sulfur springs and in sewage which contains considerable quantities of hydrogen sulfide. The sulfur granules may easily be observed by an examination of the organisms under the microscope. This oxidation of hydrogen sulfide to free sulfur and sulfuric acid enables the organism to manufacture its food by means of the energy thus secured, i.e., these organisms are autotrophic and chemosynthetic.

The production of sulfur acids under certain conditions, as in contact with concrete, may prove destructive or injurious. This has happened, for example, in some cases in the concrete tanks used for sewage disposal. On the other hand, the oxidation of sulfur in contact with insoluble phosphates (as tricalcium phosphate) in the soil may give rise to the formation of soluble phosphates, thereby enhancing soil fertility by increasing the availability of plant food.

Reduction of Sulfur Compounds. In the presence of organic matter and in the absence of oxygen, certain organisms are known to be able to reduce sulfates to sulfides. This is of importance in the disposal of sewage in localities where the water supply contains considerable quantities of sulfates. Such sewage carries organic matter in considerable quantities, and anaerobic conditions are quickly established particularly in sewage treatment chambers such as septic tanks. The hydrogen sulfide developed under those conditions may be sufficient in quantity to create a decided nuisance. Similar effects sometimes obtain when sewage is allowed to flow into sea water because of the considerable amount of sulfate contained in the latter. Organisms capable of bringing about such changes are for the most part short curved rods, particularly *Vibrio desulfuricans*. The facility with which sulfur acids may be formed under aerobic conditions from hydrogen sulfide and sulfur and the equal facility with which hydrogen sulfide may develop from sulfates under anaerobic conditions in the presence of oxidizable carbon compounds are quite significant in certain soils as, for example, in determining the life of concrete drainage tile and of iron or steel pipes laid in it.

Organic Sulfur Compounds. Sulfur enters into the makeup of many organic compounds. Sulfur amino acids as cysteine and methionine are present in most proteins. The vitamin thiamin also contains sulfur. These organic compounds are rapidly decomposed by many kinds of microorganisms, with production of hydrogen sulfide. As noted above this is transformed promptly under aerobic conditions into sulfates, in which form it is taken up by the roots of plants. These plants again reduce the sulfate and use the reduced sulfur in the synthesis of amino acids and proteins.

TRANSFORMATION OF PHOSPHORUS AND ITS COMPOUNDS

Phosphorus does not appear in soil in the free form. The phosphoric acids play a very important role in the metabolism of all living cells. The importance of the phosphorylases and the phosphatases in both breakdown and synthesis of organic compounds has already been noted. Organic compounds containing phosphorus enter the soil with barnyard manure and with all plant remains. The phosphorus is rapidly converted into free phosphoric acid, which, for the most part, promptly reacts with bases, particularly calcium, magnesium, and iron. A part is converted into relatively stable organic compounds which are generally present in the humus of fertile soils.

Phorphorus as phosphoric acid is essential to the growth of all crops. To be available to the roots it must be soluble. The phosphate present in most soils is largely in insoluble form (usually as tricalcium phosphate). Commercial fertilizers containing phosphorus usually contain either calcium phosphate, which is quite insoluble in water, or calcium acid phosphate, which is slightly soluble. The insoluble phosphates are slowly converted into soluble form available to plant roots by several processes. The roots themselves in contact with insoluble phosphates are able to dissolve them slowly probably because of the excretion of weak organic acids, such as carbonic acid. Bacteria through similar processes may bring about some solution also. In some cases the bacteria may produce stronger acids which are even more effective. Reduced sulfur compounds entering the soil are converted promptly to sulfuric acid which in turn can transform the lime phosphate to soluble acid phosphate.

In preparation of commercial acid phosphate for fertilizers it is customary to treat rock phosphate (tricalcium phosphate) with sulfuric acid which converts it into calcium acid phosphate. Lipman and coworkers found that it was possible to bring about this same change by addition of powdered free sulfur to soil. Bacteria such as *Thiobacillus thioxidans* oxidize the sulfur to sulfuric acid.

TRANSFORMATIONS OF CARBON AND ITS COMPOUNDS

Pure carbon in the form of charcoal, when added to soil will be slowly oxidized by soil microorganisms with formation of carbon dioxide. The kinds of organisms responsible and the sequence of changes whereby an organism can secure growth energy by this process of oxidation are imperfectly known. Pure cultures of the organisms and studies of their metabolism are lacking.

Coal may also be attacked by soil organisms; here again details are imperfectly known. It is known that certain fungi, members of the genus *Aspergillus*, will grow on brown lignite or even black lignite which contain numerous compounds in addition to free carbon.

The changes which are produced in various carbon compounds in the soil are quite identical with those which have been previously discussed under fermentations and basic mechanisms. There are some few types of changes particularly characteristic of the soil which require some additional discussion.

Soil has constantly added to it the excretions of all sorts of living things, the dead bodies of animals, and insects of many kinds, the leaves, stems, and roots of dead plants, including those of herbaceous plants, shrubs, and trees. Organic compounds of bewildering variety are worked over by the soil flora and fauna, and eventually by the bacteria and fungi. In large part the carbon of these organic substrates is returned to the air as carbon dioxide, the nitrogen becomes ammonia and nitrates dissolved in the soil water, and hydrogen becomes water. However, in most fertile soils, such as prairie soils to which there is added the annual increment of all the prairie vegetation, there is the gradual accumulation of considerable amounts of relatively stable organic matter or humus. Analysis of the humus shows it to be somewhat variable and also relatively complex in composition. It is certainly not a single compound, but a mixture. It contains carbon, hydrogen, oxygen, nitrogen, phosphorus, sulfur, and sometimes other elements. It is colloidal in nature and can hold many substances introduced into the soil through its powers of adsorption. It is continually being formed and decomposed. In the latter process nitrogen, phosphorus, and sulfur are put into forms which may be taken up by roots. Humus also has high absorption powers and is significant in soils in increasing their water-holding capacity. It also has much to do in the determination of the texture, friability, and water permeability of soils.

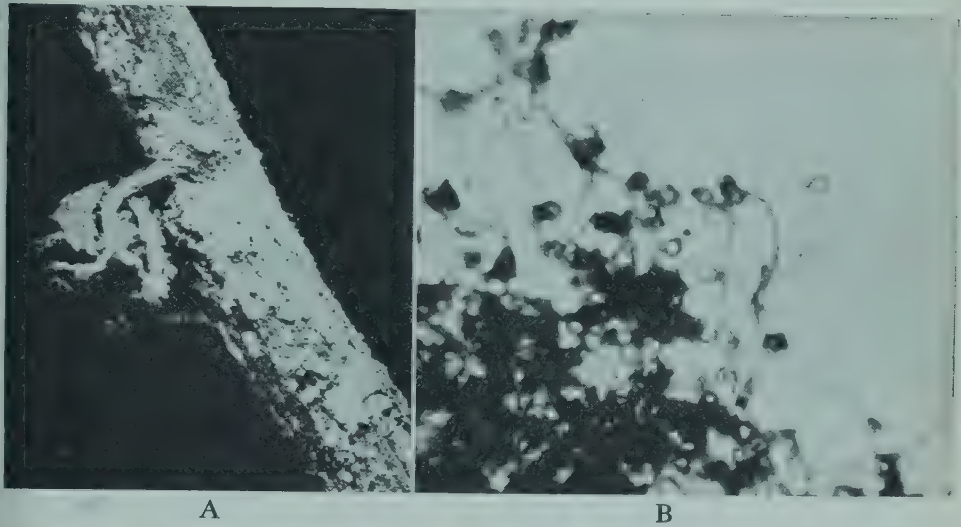

Fig. 26–3. A soil *Streptomyces*. A. Growth on the surface of a bit of alfalfa straw in the soil. B. Photomicrograph of the aerial mycelium and spiral coils of spores.

Among the materials most abundantly and constantly added to soils through plant remains are the complex plant carbohydrates lignins and similar substances relatively refractory to decay, together with chitinous coatings of insects, and the mycelium and fruiting bodies of fungi. All these rather rapidly lose their identity in the soil and soon disappear.

Cellulose is the most abundant of the materials added to soil. The chemistry of the breakdown to cellobiose and glucose has already been discussed (Chapter 22). Many organisms, snails, insects, protozoa, fungi, and bacteria have been described as capable of decomposing cellulose and utilizing the products. The protozoa and bacteria important in its digestion in herbivorous animals will be considered in Chapter 27. In soil are to be found both aerobic and anaerobic bacteria and fungi which destroy cellulose.

The genera *Cytophaga, Sporocytophaga,* and *Polyangium* of the slime bacteria (*Myxobacterales*) include several species important in decomposition of cellulose. These organisms are flexible, elongate, usually tapering rods which show a creeping motility when in contact with a solid substrate. Some species are brightly colored, forming orange, red, or yellow colonies (as *Cytophaga aurantiaca, C. rubra, C. lutea, Sporocytophaga myxococcoides*). The genera differ from each other primarily in that *Sporocytophaga* produces spherical or ellipsoidal microcysts or spores that are loosely embedded in slime among the vegetative cells. Species of *Cytophaga* do not produce spores (microcysts). In *Polyangium cellulosum* definite orange-fruiting bodies consisting of clumps of cysts are produced, the cysts containing the spherical spores formed by a shortening of the rods. The bacteria swarm over the surface of the plant material or cellulose attacked, eventually dissolving it. Little is known of any intermediate products of cellulose digestion.

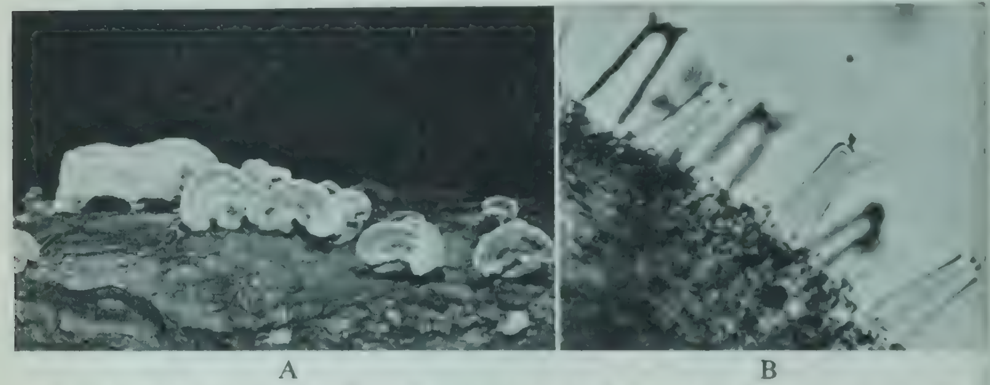
A B

Fig. 26–4. A. Fruiting bodies of one of the shelf fungi on decaying wood. The mycelium of this fungus penetrates the wood and brings about its decomposition. Basidiomycetes of this type are important in the destruction of lignin and of cellulose. B. Section through the hymenium or fruiting surface, showing the short basidia and the large cystidia or spines.

Other aerobic cellulose organisms have been described belonging to the following genera: *Cellfalcicula,* which in some respects resembles *Cytophaga* but is motile by means of a single polar flagellum; *Cellvibrio,* with elongate curved cells with single polar flagellum; *Cellulomonas,* rod-shaped with one to several peritrichous flagella.

Several molds, particularly members of the genus *Trichoderma* attack cellulose. However, certain of the higher fungi are probably much more important, including many representatives of the gill and pore fungi such as *Merulius, Polyporus, Pleurotus, Fomes,* and many others. These all belong to the *Basidiomycetes* characterized by producing spores (usually in fours) upon basidia which cover the gills or line the pores of the fruiting bodies. Frequently these gills or pores also have larger projecting bodies called *cystidia,* which are quite characteristic of the several species. There are also many species of cup fungi belonging to the *Ascomycetes* which bear their spores, usually eight in number, in elongated sacs. Among the common forms are members of the genera *Peziza* and *Bulgaria*. The mycelium of these higher fungi penetrates woody and other plant tissue, dissolving the cellulose and lignin present. They are common agencies in causing rotting and decay of wood.

There are also anaerobic bacteria which are active in cellulose decomposition. Some of these are thermophilic, growing at temperatures as high as 65° C. They are spore-bearing bacteria belonging to the genus *Clostridium*.

CHAPTER 27

Microorganisms as Symbionts and Commensals in Animals

The fact that the substances produced by one organism may be of great significance in the growth of another, that microorganisms have many interrelationships with the other living plants and animals of their environment, has already been discussed briefly. These interrelationships have been denoted by such terms as symbiosis, antibiosis, metabiosis, commensalism, and synergism. Previously discussed have been the production of antibiotics, the symbiosis of bacteria with the legume in the nodule, the sequence of changes that occur in the production of butter flavor. There yet remains for consideration certain interrelationships between microorganisms and animals. Of special interest are certain of these in the insects and in higher animals.

Microorganisms Symbiotic with Insects. Many insects harbor microorganisms which are believed to be symbiotic. In many forms the microorganisms are to be found in certain differentiated structures or organs of the body. In some cases these are enlarged body cells termed *mycetocytes,* in other cases a special multicellular organ is developed, a *mycetoma,* in which they live: such, for example, are the rather conspicuous *fat bodies* that are evident in some insects. There seems in some cases to have been developed a most intimate interrelationship between the insect and the microorganism. It has been shown, for example, that in some insects there is provision whereby each egg laid is supplied with an inoculum of the organism, which enters through the micropyle and participates in the early organization of the mycetomata. In some insects more than one type of differentiated organ with its symbionts have been found, each with its special kind of microorganism. There is increasing evidence that some at least of these symbionts perform the function of synthesizing vitamins essential to the growth of the insect.

Microorganisms in some cases are essential to the life of the insect through participation in the digestive functions. There is evidence that some insects which live upon wood, such as wood borers, have intestinal bacteria that are of significance in the digestion of the ingested wood. In some cases the microorganisms are protozoa, as in the termites. It has been shown that the protozoa in the intestines of these insects themselves take up and digest cellulose, the products of digestion being directly or indirectly available to the insect as food.

Great difficulty has been encountered in securing cultures of these symbiotic microorganisms which may be grown outside the body of the insect. Morphologically, some of these symbionts seem to be bacteria, some yeasts, some fungi and some protozoa.

Role of Microorganisms in the Alimentary Tract of Higher Animals. Bacteria, yeasts, and frequently protozoa are to be found normally in the alimentary tracts of most animals. In some they apparently play an important role in digestion of certain foods and the synthesis of amino acids and vitamins. Their importance in ruminants was emphasized more than a hundred years ago by two French students, Gruby and Delafond, who stated:

> From the very great number of these animalcules in the first two stomachs of the ruminant, the presence of their shells in the third and fourth stomachs and in the excreta . . . we are forced to conclude that the organic matter of these animalcules is digested in the fourth stomach of the ruminant.

Digestion in animals such as the cow, sheep, and goat (the ruminants) is apparently greatly facilitated by the presence of certain microorganisms in the paunch (rumen or first stomach). The rumen and reticulum constitute a relatively large fermentative and preliminary treatment chamber in which a good deal of predigestion occurs. The rumen of a cow may have a capacity of forty to fifty gallons. The coarse food, hay, and grain are swallowed after partial mastication. After storage in the rumen for a time, the food is returned to the mouth in small masses, where it is more thoroughly chewed and again swallowed. The rumen is ordinarily about half filled by liquid. The amount of liquid is maintained at a fairly uniform level by the water drunk and by the relatively large amounts of saliva formed by the salivary glands and passed in a steady flow to the rumen. Unlike the saliva of man and many animals, this saliva contains very little or no digestive enzyme, but is buffered at pH 8.2 by sodium bicarbonate.

A cow may secrete as many as forty or more liters of saliva per day. The contents of the rumen are constantly buffered and are prevented by the sodium carbonate from becoming too acid. The rumen contents are continually mixed and agitated by contraction of the muscles of the rumen walls, and this tends to force the liquid over the surface of the food. Eventually, the partially digested food is passed into the next compartment of the alimentary tract, the omasum.

Examination of the rumen contents makes it evident that there are vigorous fermentative changes going on. Acids are produced, likewise large quantities of gas. After ingestion of a considerable quantity of food, as many as sixty to one hundred liters of gas may be produced by a cow in a few hours. Most of the gas is methane, with some carbon dioxide, and with some traces of hydrogen sulfide. Normally the gas passes by way of the esophagus to the mouth and escapes. Belching of this gas is a constant and regular physiologic function in the cow. Under conditions, not very exactly known, particularly after ingestion of certain feeds, there may be an occlusion of the esophagus, with consequent failure of the normal belching process. The gas then accumulates and distends the rumen, producing the disease *bloat*. If not relieved by resumption of belching or by puncture through the body and paunch walls, a severe case of bloat will prove fatal. There have been many theories advanced to explain the loss of ability to

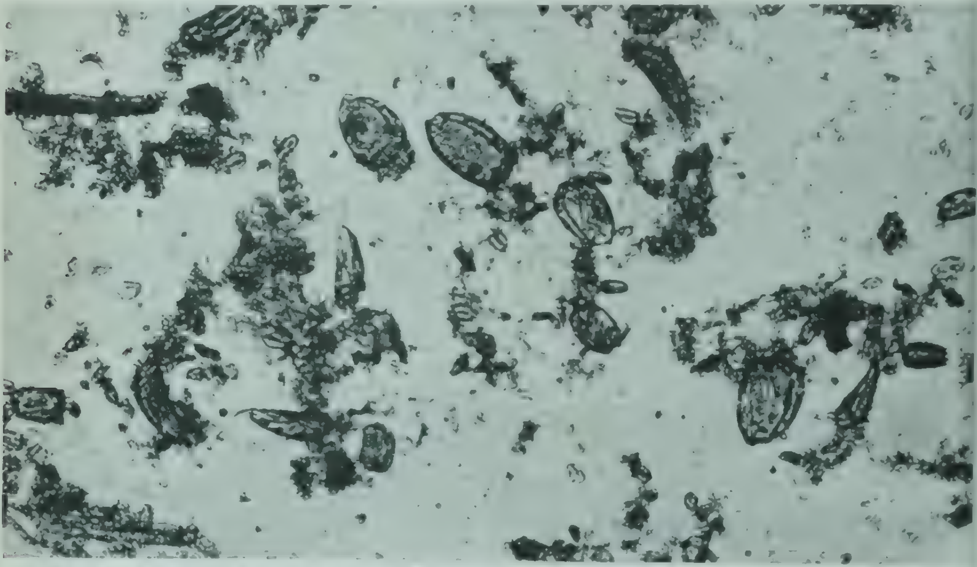

Fig. 27–1. Bacteria, yeasts, and protozoa, together with fragments of fodder in a drop of liquid taken from the rumen of a cow.

belch and the consequent development of bloat. Character of the feed, its succulence, the absence of coarse irritating material, and the inherent reflexes of the animal are all claimed to play a part.

Microscopic examination of the liquid contents of the rumen reveals the presence of great numbers of bacteria, yeasts, and protozoa. It is evident that the rumen is a great fermentation vat. All the changes which occur have not as yet been satisfactorily explained, but enough is known to show the significance of many of the organisms present.

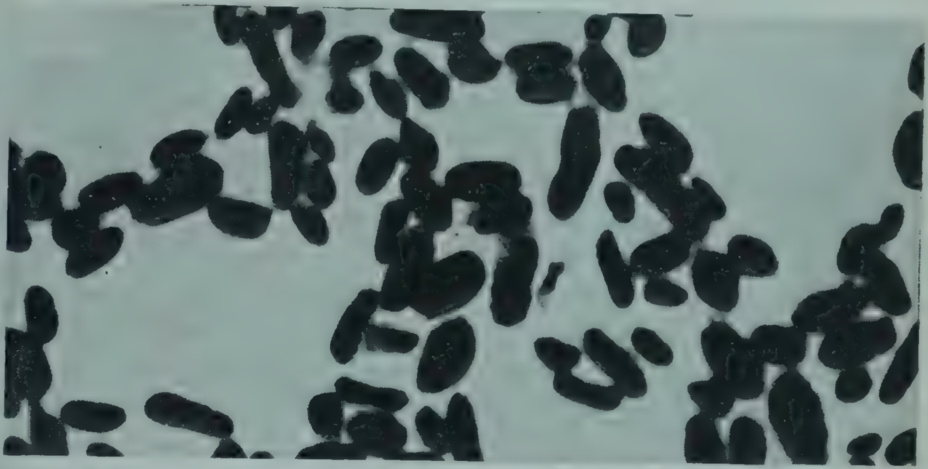

Fig. 27–2. Yeasts cultivated from the contents of the rumen of the cow. Photomicrograph.

The formation of gas and acid is due largely to the activity of enormous numbers of bacteria. Some of these bacteria are apparently of the types known to produce lactic and acetic acids under anaerobic conditions. What organisms are responsible for the production of the great quantities of marsh gas or methane is quite uncertain, although it is probable that the same types of organisms and the same processes described under methane fermentation are active. Comparison of the organisms secured by use of the common bacteriological techniques with the numbers visible microscopically makes it evident that relatively few have been cultivated and that little is known relative to some of the most important species present.

However, while relatively little is known of the individual species present, a good deal has been discovered about the changes they produce. Studies at the Illinois Agricultural Experiment Station by Johnson and others have shown that by appropriate centrifugation of the liquid contents of the rumen, a mass of bacteria may be secured with a 45 per cent content of protein. These rumen bacteria when fed to laboratory animals were found to have digestive coefficients of about 55 per cent. The relatively low digestibility of the protein was believed to be due to the fact that the bacteria had not been completely freed from particles of feed. The biological value of the proteins was good. It has been concluded, therefore, that much, if not most, of the nitrogenous constituents of the feed of cattle are digested and assimilated by the bacteria (and as noted below, by the protozoa) of the rumen. In turn, these bacteria in part are destroyed and digested in lower parts of the alimentary tract (and the protein digestion products assimilated), and in part ingested and digested by the protozoa present. Just what proportion of the protein of the feed is thus "worked over" by the bacteria and what part passes on for direct digestion by the animal is not certainly known.

Many kinds of bacteria (not all) are able to synthesize their own proteins if furnished ammonia together with a suitable carbon source such as a carbohydrate. This ability to utilize ammonia is widespread, almost universal in plants; in fact, the ability to utilize ammonia in nitrogen synthesis has long been regarded as a good criterion for the differentiation of most plants from animals. The latter, in general, utilize amino acids as their simplest nitrogen source. If ammonia or similar simple nitrogen compounds are fed to a ruminant, are the bacteria present in the rumen able to synthesize protein from it and thus make such "inorganic" nitrogen available to the animal? The answer seems to be yes. Studies have been made extensively both with ammonium salts and with urea added to cattle rations. It has been shown that a considerable proportion of the nitrogen needs of the animals may be met with nitrogen from these sources. When urea is added to liquid taken from the rumen, it is found to be converted promptly into ammonia because of the presence of considerable amounts of the enzyme *urease*, apparently produced by the microorganisms present. The reaction consists essentially in the hydrolysis of the urea to ammonia and carbon dioxide:

$$(NH_2)_2CO + H_2O \xrightarrow{\text{Urease}} 2NH_3 + CO_2$$

Studies carried out on nutrition of cattle, including dairy cows, have shown that urea may be substituted to some extent for the more expensive protein nitrogen, and dependence may be placed upon the activities of the rumen organisms to make it useful to the animal. Ammonia and urea can be made in large quantities synthetically and at prices which make urea as a constituent of cattle feed competitive with the feed proteins. Commercially mixed feeds containing urea were used in 1944 to the extent of 100,000 tons. Experience has shown that the urea content of the feed should not exceed 3 per cent of the grain. Large amounts are toxic.

Studies at the Cornell Agricultural Experiment Station have shown that the ten amino acids usually regarded as essential to growth in mammals are synthesized in large amounts in the rumina of animals fed urea as the only dietary source of nitrogen. The hydrolysate of the synthesized proteins showed the presence of the amino acids arginine, histidine, isoleucine, leucine, lysine, methionine, phenylalanine, threonine, tryptophan, and valine. Various microorganisms, particularly bacteria and yeasts, are able to synthesize vitamins. This is particularly true of various members of the vitamin B complex, among them thiamin, riboflavin, nicotinic acid, pantothenic acid, pyridoxine, and biotin. The syntheses of all have not been completely demonstrated. The Wisconsin Experiment Station has shown that the addition of urea to the ration increased the synthesis of riboflavin, nicotinic acid, biotin, and pantothenic acid when suitable carbohydrates are present. These rumen-produced vitamins are in part taken up by the animal, some secreted with the milk. A part apparently passes through the alimentary tract, for it has been shown that cow manure may be a good source of vitamins for poultry.

The extent to which the bacteria directly attack cellulose and similar fibrous material in the rumen contents is not well understood. The fact that methane formation is a common concomitant of cellulose destruction by bacteria may indicate that there is considerable digestion. It is, of course, well known that ruminants regularly digest a portion of the cellulose which they ingest, though there is no evidence that any cellulose-digesting enzyme is produced by the animal. Intermediate products of digestion such as the sugars and acids would be absorbed and utilized by the animal.

The protozoa constitute a most interesting part of the rumen contents. Many genera and species have been described which have not been found in nature outside of the rumen. They are not absolutely essential to the nutrition of the animal, for Becker and others working with goats found it possible to get rid of all the conspicuous protozoa of the rumen by use of disinfectants such as copper salts. Apparently these protozoa do not occur commonly in hay and other feed materials, for animals which had been *defaunated*, that is, animals from which the protozoa had been removed, did not become reinfested even though their feed was not sterilized. It is probable that infestation is directly from animal to animal by way of the mouth. Animals thus defaunated remained healthy, so that the protozoa evidently are not essential. This does not mean, of course, that they do not regularly play a role in the changes produced in the normal animal. They are frequently present in enormous numbers, even one-half to two million per milliliter. They may constitute as much as one-fifth of the dry matter of the rumen contents. The numbers are greatly influenced by the amount and character of the feed consumed by the animal.

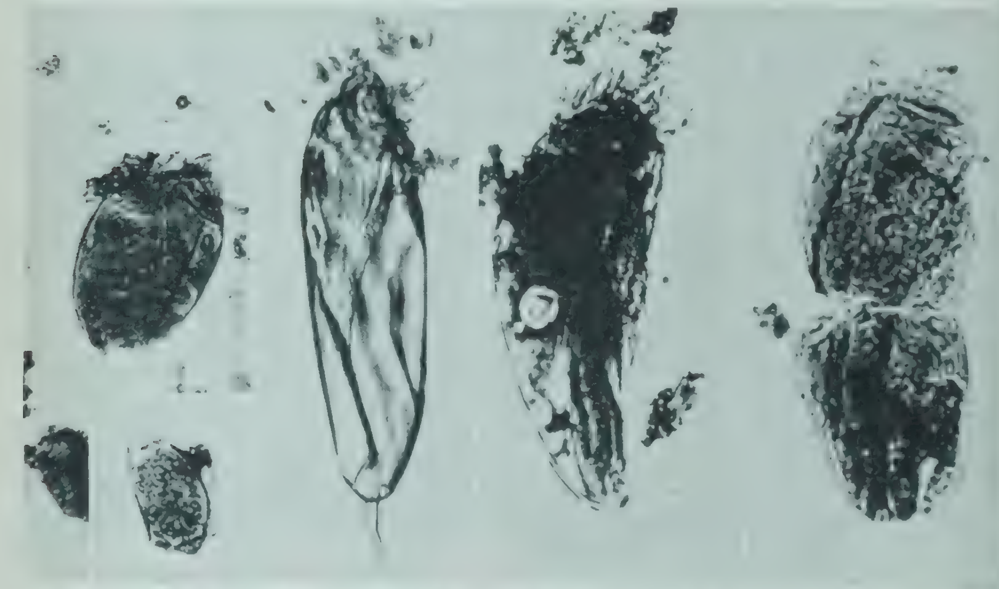

Fig. 27–3. Photomicrographs of several of the types of protozoa commonly found in the rumen of the cow. These relatively complex organisms are all unicellular. They are active in the ingestion of the bacteria present and probably also function in the digestion of the cellulose.

The larger and more conspicuous of the protozoa are ciliates. Some of them are among the most differentiated of unicellular animals. The cilia are in rapid motion, causing a streaming of liquid containing bacteria and particles of hay, grass, starch, and similar materials into the oral groove. These are then passed on into the cell itself, where they are digested and built up by the cell into protoplasm and cell organella. They are anaerobic, as might be expected of organisms which grow in a medium in which methane is being rapidly evolved. Johnson and others at the Illinois Station separated the protozoa from rumen contents by washing, filtration, and decantation, securing a mass relatively free of feed particles and bacteria. The protozoa when dried were found to contain nearly 55 per cent crude protein which when fed to rats showed a true digestion coefficient of about 86 and a biological value of 68.

Hungate (1942) showed that it is possible to cultivate certain of these protozoa outside the rumen. A species of *Eudiplodinium* was grown anaerobically for almost two years in a medium containing inorganic salts, pulverized dried grass, cellulose and bacteria.

CHAPTER 28

Disease: Its Meaning in Animals and Plants

The term *disease* may be defined in various ways. A convenient statement is that disease is the converse of health; it is a condition resulting from the malfunctioning of the body of a plant or animal or some one or more of its parts or organs. Such a definition is somewhat inexact, but sufficient for our present need.

So far as they have been studied, practically all kinds of animals, including man and plants, suffer from abnormal functioning; they are sometimes diseased. Observations were first made upon diseases of man, then of animals, and later, of plants.

Several theories of disease have been held successively during ancient, medieval, and modern times. For example, according to the *demonic* theory, evil spirits were believed to invade the body. Disease was sometimes attributed to the direct displeasure of the gods. Exorcisms, spells, amulets, charms, etc., were commonly used to avert disease, in fact, their use has not entirely disappeared among supposedly civilized people.

The ancient Greek Hippocrates, generally termed the father of medicine, developed the so-called *humoral* theory of disease which held sway until comparatively recent times. According to his theory the body contained four humors: blood, phlegm, yellow bile, and black bile. When these were mixed in the proper proportions, the body was considered to be in a state of health. When one gained the ascendancy and there was an improper mixture, the body was believed to be diseased. Many of the methods of treatment used by physicians in olden times, such as bloodletting, were based upon this theory. This theory began to break down in the sixteenth century, and during the next two centuries many others were advanced to take its place.

About the middle of the nineteenth century Murchison, an Englishman, suggested that disease always originates in dirt or filth of some kind. Disease-producing organisms were supposed to originate *de novo* in such material, at least they found there favorable conditions for their multiplication. Heaps of decaying organic matter were held to be centers of distribution for harmful microorganisms. Considerable emphasis was likewise laid upon the ability of organisms to be carried through the air by sewer gas and by the gases (*effluvia*) rising from swamps. Later, this theory was somewhat modified and was finally regarded as an explanation of the spread of certain of the fevers. Murchison's definition of typhoid fever is:

> an endemic disease generated and propagated by certain forms of decomposing matter. It may be generated independently of a previous case by fermentation of fecal and perhaps other organic matter. It may be communicated by the sick to persons in health, but even then, the poison is not like that of smallpox, given off from the body in virulent form, but is developed by the decomposition of the excreta after their discharge. Consequently an outbreak of typhoid implies poisoning of air, water, and other ingesta with the decomposing excrement.

Emphasis should be laid upon the fact that he believed diseases of this type could originate without any preexisting disease of the same type to serve as a source of the virus.

The theory of disease which is now commonly held and which is the only one which rests upon a firm foundation of fact is the *germ* theory. The researches of Louis Pasteur upon the causes of various types of fermentation, such as the production of alcohol and carbon dioxide by yeasts from certain sugars and the transformation of the lactose in milk to lactic acid by bacteria, suggested that specific organisms might, in somewhat analogous manner, produce disease in man and animals. Early in the nineteenth century the disease known as *favus,* a kind of ringworm of the scalp, was shown to be due to a specific fungus. About the same time a silkworm disease was likewise found to be caused by a parasitic fungus. Since about 1875 the specific causes of a great many of the diseases of man, animals, and plants have been determined, although there are still some which thus far defy the efforts of investigators. The germ theory of disease does *not* insist that all diseases are caused by microorganisms. There are many diseases in which the cause is a constitutional or hereditary defect of some kind. Diabetes, epilepsy, rickets, and dietary deficiency diseases in general are certainly not caused in any direct way by microorganisms.

The channel through which a disease-producing organism gains entrance into the body is termed its channel of infection (*infection atrium*). Organisms may enter through the alimentary tract, the respiratory tract, through the skin by means of wounds, or through the skin glands. Some of the most important diseases are transmitted by ingestion, the carrier of the infection ordinarily being water or food. Diseases of the intestinal tract, such as typhoid, dysentery, and asiatic cholera, are universally acquired by this means, and some others, such as tuberculosis, may be contracted in this manner. Air usually contains bacteria, yeasts, and molds. Only under exceptional conditions are these forms pathogenic. Certain organisms, however, when breathed in with the air may gain a foothold on the mucous membranes of the respiratory tract and produce disease, as in tuberculosis. Many of the diseases at one time supposed to be transmitted by the inhalation of microorganisms have been shown to be acquired through ingestion. A few disease organisms may attack through the healthy uninjured skin. Among them are certain of the molds producing ringworm and similar diseases. A few disease organisms, too, enter by direct inoculation into the body. Tetanus, or lockjaw, is acquired by the introduction of the organism into a wound and rarely in any other way. Certain diseases are transmitted only by insects. Malaria and yellow fever, for example, are never transmitted otherwise than by direct inoculation into the body by mosquitoes. Certain blood-sucking flies transmit other diseases, such as the sleeping sickness of man and related diseases of animals in Africa and in tropical countries. Flies may also serve as carriers of disease-producing organisms from the filth in which they breed to food used for human consumption.

Classification of Diseases. Diseases as noted above may be divided into two groups, those caused by parasitic microorganisms (*infectious diseases*) and those not so caused (*non-infectious diseases*). Popularly the term *infectious* is often used loosely to indicate the method of transmission of a disease. Literally, it has nothing directly to do with such transmission. A considerable proportion of the diseases known are infectious. Such, for example, are erysipelas, diphtheria, typhoid fever, smallpox, Asiatic cholera, bubonic plague, whooping cough, dysentery, tuberculosis, pneumonia, anthrax, and tetanus. An individual harboring a disease-producing organism is said to be *infected* by the organism. Any article of clothing or inorganic material of any kind which may serve to harbor the organism and aid in the transmission of the disease is said to be *infective*.

Transmissible Diseases. Diseases caused by microorganisms may be subdivided on the basis of the ease with which they may be transmitted from one individual to another. A *contagious* disease is one that is readily transmitted by direct or indirect contact between a diseased individual and one who is healthy. A non-contagious disease is one which is not readily transmitted in this manner. All gradations may be found between these two classes of diseases, for we speak of some diseases as being highly contagious, others as mildly contagious. Relatively few of the infectious diseases are quite non-contagious. They are, for the most part, those diseases which require direct inoculation for their transference, such as tetanus or lockjaw, malaria, and yellow fever.

Disease Types Produced by Microorganisms. The changes brought about by the growth of disease-producing organisms in the body are quite varied, differing quite as much as the morphologic and cultural characters of the organisms when grown outside of the body. Certain classifications of types of diseases, however, prove useful in discussion.

A disease caused by a single species of organism working alone in the body may be termed a *primary* infection. Sometimes two or more organisms may be found associated together in producing disease. Such would be termed a *mixed* infection. In some cases, infection with one species of organism may so weaken the resistance of the body that it readily becomes a prey to some other disease. For example, pneumonia not infrequently accompanies or follows typhoid fever or measles. The organism causing pneumonia is distinct from those causing the other diseases, but finds opportunity for development when the body has become weakened. Such a disease is said to be the result of a *secondary* infection.

Diseases are also classified by the bacteriologist and pathologist into several groups depending upon the location of the organism in the body, production of poisons or toxins, and distribution through the tissues. A *toxemia* is a condition in which the organism remains localized and produces a poison or toxin which enters the blood stream and causes damage to tissues in other parts of the body. For example, the diphtheria organisms rarely leave the throat but produce a toxin which enters the blood stream and causes damage to the heart muscles, the liver, and other tissues. In certain diseases or infections the organisms remain localized but do not form an appreciable amount of toxin. Such are wound infections and local inflammations in general. When the blood stream is invaded, the infection may be termed a *bacteremia*. Such general invasion may occur in typhoid fever. The term *septicemia* is frequently used synonymously with bacteremia, although some authors limit it to those diseases caused primarily by pus-producing organisms in which the bacteria multiply in the blood stream. A *sapremia* is a disease produced by the absorption of poisonous putrefactive products from dead or decaying tissues. Such might result from gangrene or from a retained placenta. A disease in which there is an eruption of the skin, as smallpox or chickenpox, is termed an *exanthema* (plural *exanthemata*).

Animal Inoculation in the Bacteriological Laboratory. Use of animals experimentally is often necessary in bacteriology. Such use is not a matter of choice with the bacteriologist or pathologist, for there appears to be no other way open for the determination of certain facts. The most important reasons for the use of animals are: (1) Inoculation of animals is sometimes necessary to recognize or *diagnose* certain diseases. For example, one of the most reliable means of determining whether or not a dog has rabies is by the injection of a small portion of the brain tissue into a rabbit. The outcome of such an injection may determine the treatment of individuals that have been bitten. (2) It is necessary in the initial study of a disease to inject organisms suspected of causing the disease into animals to prove or disprove such relationship. Had it not been for animal inoculation, it would have been impossible to have determined the causes of such diseases as tetanus and tuberculosis. (3) It is sometimes necessary to use the animal body as a kind of filter by inoculation of a mixture of organisms into the animal to eliminate all the forms incapable of producing disease. For example, in the isolation of the bacillus of tuberculosis from milk, the simplest method is to inject some of the sediment secured from the milk into a suitable animal, such as the guinea pig, and allow the disease to develop. Later when the animal is killed, the organism may be found free of other organisms in the nodules characteristic of the disease, and from these pure cultures may be more readily secured. (4) Animals are used for the manufacture of certain products designed for the treatment and prevention of disease, such as antitoxins and other antisera. These will be discussed at greater length in the next chapter. (5) Animals are essential in testing certain biological products. The only method that has been evolved for determining the strength of toxins and antitoxins is by animal injection. The chemist has thus far been unable to identify and isolate these materials. The animal is used by the bacteriologist in the laboratory in much the same manner as the chemist makes use of an indicator to determine the acidity or alkalinity of a solution.

The animals most commonly utilized in the bacteriological laboratory are the guinea pig, rabbit, mouse and rat, and more recently, the hamster. In the investigation of certain diseases, particularly those infecting birds and fowls, the pigeon and domestic fowl are used. Monkeys have been utilized for the study of some human diseases which cannot be communicated to any of the other lower animals. Calves are used for the preparation of smallpox vaccines. The horse is the common source of antitoxins.

Inoculation of animals is usually accomplished by the use of the hypodermic syringe and needle. The material may be injected under the skin (*subcutaneously*), into the veins (*intravenously*), or into the abdominal or peritoneal cavity (*intraperitoneally*). Occasionally organisms may be injected into the thoracic cavity or into the brain (*subdurally*). Animals may be infected also by causing them to *ingest* or to *inhale* the microorganisms being studied.

Koch's Postulates. Dr. Robert Koch, the eminent German bacteriologist, formulated certain rules that are followed whenever possible in the determination of the causal relationship between a specific organism and a particular disease. Koch's postulates may be enumerated as follows: (1) The organism suspected of causing the disease must be found in all cases of the disease. (2) This organism must be isolated from the diseased individual and grown in pure culture. (3) Pure cultures of the organism when injected into a suitable animal must reproduce the disease. (4) The specific organism in question must again be isolated from the diseased individual. It is not always possible to fulfill all these postulates. For example, there are diseases of man which have thus far not been communicated to lower animals, hence the third postulate cannot be satisfied. Then again there are the organisms, such as the viruses, that are ultramicroscopic, are obligate parasites, and have never been grown on culture media.

Many techniques involving serologic methods and cultivation in living tissues have been used to assist in determination of host-parasite relationships in difficult cases.

Microorganisms Normal to the Body. Before discussing the bacteria which are capable of producing specific diseases, note should be taken of some organisms which are normally present and which are not to be confused with disease-producing forms. Those normally present on plants will be discussed later. Here primary attention will be paid to microorganisms as related to man.

Many species of bacteria are known which develop on the skin and in the various body cavities which open to the surface. The tissues of the body are normally sterile. Bacteria are not usually present in the blood stream, the muscles, or glands of normal individuals, although the lymph nodes lying near the intestines may occasionally contain intestinal bacteria.

Organisms may or may not be present in the tissues of diseased individuals. It has been noted that some diseases are classed as septicemias and bacteremias because of the general distribution of the organisms through the body in the blood stream. In other cases the organisms are localized and do not get far from the original site of infection.

Flora of the Skin. Two groups of skin microorganisms may be differentiated, those whose presence is merely accidental, and those which are quite constantly present and evidently multiply upon the body surfaces. Whenever the skin comes in contact with dirt or dust or any object coated with bacteria or containing them, organisms may adhere. The bacteria of the soil are quite common upon the skin, particularly under the fingernails and in the hair. These include such aerobic forms as the organisms belonging to the hay bacillus or *Bacillus subtilis* group, and certain anaerobic bacteria. Organisms characteristic of the intestines, such as *Escherichia coli*, are not uncommon. Winslow has shown *E. coli* to be present on the hands of about 9 per cent of one hundred eleven individuals examined. Pathogenic bacteria may be present upon the skin of one who comes in contact with diseased individuals. The most common normal inhabitants of the skin, penetrating to the lower layers of the epidermis, are certain members of the genus *Micrococcus*. *Micrococcus aureus* and *M. albus* are quite commonly present, though usually lacking in virulence. These are the organisms likewise found in wound infections, abscesses, and boils. Streptococci are also frequently isolated from the skin, though more rarely than the preceding. Wherever the skin is continually moist and greasy, as in the axillae of the body, *Mycobacterium smegmatis,* an acid-fast organism closely resembling *M. tuberculosis,* is usually present. This organism does not seem to penetrate the skin, but to live in the skin secretions. The *Micrococcus cereus* is generally found in the wax secreted in the external auditory canal. The exposed mucous membrane of the conjunctiva shows relatively few bacteria in health. The secretion of tears seems to remove and perhaps also to destroy organisms that may accidentally gain entrance.

The Flora of the Mouth. A large number of bacteria have been described from the mouth. Many of these forms doubtless are accidental, but the normal mouth has a constant flora including a considerable number of species. These were first studied extensively by Müller, who decided that six organisms were practically always present. These organisms were in part relatively long threads (*Leptotrichia*) and slender spirals (*Spirochaeta*). He did not succeed in cultivating any of these. These organisms are obligate anaerobes when grown in culture media, but find favorable conditions for growth between the teeth and in teeth that are decaying. They are actively motile and are frequently to be observed moving about rapidly when a little of the tartar or material from between the teeth or lying between the gums and the teeth is suspended in physiological salt solution and examined under the microscope. Some five or six species of spirochetes have been recognized as relatively common. Certain anaerobic bacilli, some of them related to the *Clostridium putrificum* already described, have been noted in decaying teeth. Among the cocci, certain streptococci, in large part non-virulent varieties of *Streptococcus pyogenes,* are almost always present. The same is true of *Micrococcus aureus* and *M. albus. Diplococcus pneumoniae* (*pneumococcus*) can frequently be observed in stained mounts of sputum of healthy individuals. Such organisms probably are avirulent forms in most cases. Species of the genus *Lactobacillus* are particularly common; some are probably associated with dental caries. The decay of teeth has been ascribed to the development of acids by these organisms. This results in the more or less complete removal of the lime present, while the decomposition of the remaining substances of the teeth probably is brought about by common anaerobic bacteria. A foul breath is commonly due to lack of careful cleaning of the teeth and mouth. The putrefactive bacteria bring about decomposition of particles of food retained within the mouth and caught between the teeth.

The saliva seems to exert very little bactericidal action. The breath, except when forcibly expired as in sneezing and coughing, is free from bacteria as it leaves the mouth or nose.

Occasionally the mouth of a seemingly healthy individual may harbor (as in a carrier) some disease-producing organisms.

Flora of the Alimentary Tract. The alimentary tract is sterile at birth, but a few hours later bacteria are found to be present. The feces of animals living in polar regions have been shown to be almost entirely devoid of bacteria. Young guinea pigs removed by Caesarean section from the mother have been kept alive for considerable time under sterile conditions. Some insects (as the cockroach) may be reared through many generations without bacteria in the intestines. On the other hand, an effort to raise chicks without bacterial infection has resulted in failure. The fact that microorganisms in the alimentary tract are essential to normal development of many animals and insects has been discussed in Chapter 27.

Stomach. Bacteria do not normally develop in the human stomach, owing to the acidity of the gastric juice. When this acidity is diminished for any reason, bacteria may multiply. Organisms belonging to the butyric acid group and *Aerobacter aerogenes* may produce an active fermentation of the food taken into the stomach with the development of gas and other products of decomposition.

Intestines. Bacteria are relatively sparse in the first portion of the small intestine (the duodenum). As the food passes along the intestines, however, the acidity is neutralized and conditions are rendered favorable for bacterial growth. Certain of the digestive juices, particularly bile, are markedly antiseptic toward some microorganisms and more or less completely inhibit their growth. This is not true, however, of the normal flora of the intestines. In the lower part of the small intestines, or ilium, bacteria become more numerous. *Aerobacter aerogenes* is present and *Escherichia coli* in smaller numbers. In the colon the bacteria reach their maximum development. *A. aerogenes* and *E. coli* are practically always present, the latter in great numbers. These gram-negative forms in addition to the gram-positive *Lactobacillus bifidus* and *L. acidophilus* and related forms are believed to inhibit the growth of many other species, particularly the putrefactive forms, by the formation of acid and possibly of other metabolic products. Certain anaerobic spore-producing forms, particularly *Clostridium putrificum* and *C. welchii* already described, are also quite constantly present. They rarely gain the ascendancy in the healthy normal intestinal tract. It is probable that in nonruminant herbivorous animals organisms which are capable of hydrolyzing cellulose may be of assistance in the digestion of the food. Such cellulose-digesting forms have been isolated from horse dung.

Human feces contain relatively enormous numbers of bacteria. Frequently from one-fourth to one-third of the dry weight of the fecal material consists of the bodies of microorganisms. By no means all of these will develop upon artificial media. Probably in part they have been destroyed by their own products of metabolism, and in part they do not develop because suitable culture media are not used. There are usually present in each gram of human fecal material between one million and forty million living bacteria which will develop upon artificial media. The changes due to putrefactive bacteria in the intestines have been emphasized within recent years as being of considerable importance. It is believed that the products of decomposition, when these organisms are in the ascendancy in the colon, are absorbed in part through the intestinal wall and appear in the same form or as related compounds in the urine. It has been contended by some investigators that certain of the changes characteristic of old age are due to these putrefactive substances. Metchnikoff and his co-workers believed that these putrefactive organisms may be supplanted in the colon as a result of continued use of foods or beverages containing large numbers of lactic acid bacteria. The putrefactive forms do not develop in the presence of lactic acid or of the lactic acid bacteria. The organism which was advocated for this purpose was the *Lactobacillus bulgaricus,* the specific cause of the souring characteristic of Bulgarian soured milk or joghurt. The *L. acidophilus* has been found to be better adapted to this purpose. Enough evidence has been gathered to emphasize the need of a maintenance of a proper bacterial flora in the intestines, particularly in the colon, if the general health of the body is to be conserved. That the character of this flora may be determined by the diet seems to be demonstrated. However, the proof that there is any direct cause and effect relationship between the flora of the alimentary tract and the symptoms of old age is quite inadequate.

Flora of the Respiratory Tract. The nose is an efficient instrument for catching dust particles, bacteria, etc., which may be present in the inspired air. The nose can scarcely be said to have a characteristic flora, but the organisms present in the air may be usually isolated from it. By the time the air reaches the bronchi, it is largely freed from organisms and only rarely do such penetrate to the bronchioles and alveoli of the lungs. These latter tissues are usually entirely free from organisms. Those particles of dust and those microorganisms that fall upon the surface of the mucous membranes are caught by the sticky mucus which is secreted and forced to pass in the direction of the mouth by the ciliated epithelium. This constitutes a kind of drainage system for the elimination of all such materials.

Flora of the Genito-Urinary System. The exposed mucous surfaces of the urinary meatus and of the vagina usually harbor certain bacteria. For the most part these are harmless, but cocci identical with, or closely related to, the pus-producing cocci are commonly present. The secretions of the vagina are believed to be somewhat bactericidal, but certain bacteria are found quite constantly. It has been claimed that the characteristic properties of this secretion are in part due to the organisms present.

Bacteria do not normally develop within the uterus, although they are not uncommon in the later stages of pregnancy. The urinary bladder is normally sterile.

In the smegma about the external genitals acid-fast bacteria, particularly *Mycobacterium smegmatis*, are quite constantly present. This latter must be carefully differentiated by the diagnostician from the bacillus of tuberculosis which it closely resembles.

CHAPTER 29

Resistance to Disease: Immunology and Serology

The resistance to infection which is offered by the body is termed *immunity*. Certain facts of immunity are familiar to all. It is well understood, for example, that an attack of a disease such as smallpox renders the individual relatively *immune* or quite resistant to subsequent exposure to that disease. Many of the diseases attacking the lower animals are not communicable to man, i.e., man is immune to them. The converse of immunity, that is, the lack of resistance to disease, is termed *susceptibility*.

All gradations between complete immunity and high susceptibility may be observed. Morbidity (sickness, display of symptoms of disease) is an indication of susceptibility, whether the attack of the disease results in recovery or death.

All the symptoms of a disease taken together, i.e., the overall picture of a disease, is termed its *syndrome,* or more accurately, is *pathological syndrome*.

The effect of any attacking organism upon the host, that is, whether or not disease will result, depends upon the interplay of three sets of factors:

1. The inheritance of the host, that is, the genes which govern the development and physiology.
2. The inheritance of the invading organism, that is, its genic make-up.
3. The character of the environment.

Relative immunity, resistance, and susceptibility all are to be attributed to these complexes. They are to be considered in some detail.

In the interaction between host and attacking organism it should be remembered that one is frequently concerned with populations and not merely with individuals. One population of animals through inbreeding and selection may have become relatively resistant, another by a similar process may be uniformly quite susceptible, in still another the population may be unselected and the individuals may exhibit all levels of susceptibility. Similarly with the attacking microorganism, the population may be uniformly virulent, or uniformly avirulent or mixed. Obviously nine combinations of host resistance and infecting agent virulence are possible, with consequent differing results.

Inheritance and Disease. Certain pathological conditions are inherited; they are due to the presence of genes which bring about abnormalities, some of them fatal. The lethal gene may exhibit its effect by destroying the animal while an embryo. It may be a dominant and show its effect on all individuals, as is the case with certain diseases in animals such as epilepsy in cattle and the creeper type of domestic fowl in which the wings and legs are much reduced. In other and much more common cases the gene action is recessive and there is usually evidence of disease only when inherited from both parents. Dozens of diseases of this type have been described, such as dwarfing in the mouse and rat and hairlessness in the cat and the pig. The inherited defects that have been studied in man number over two hundred, and for the fruit fly (*Drosophila*), so extensively used in genetic studies, several thousand have been recorded.

In inherited abnormalities the presence of a gene which is injurious in its influence makes it permissible to designate such a gene as the etiological agent in a certain disease quite as truly as pathogenic bacteria may be so designated.

Inasmuch as inheritance must come through the germ plasm, and must be controlled by genes, and since pathogenic organisms in the nature of the case cannot be genes, an infectious disease can never be inherited. There are, however, cases of transmissions of infectious diseases, in which the individual is diseased at birth. Such cases do not, however, constitute exceptions to the rule. Salmonellosis (so-called pullorum disease) is transmitted through the egg to the chick, but the disease is in no sense inherited. In this disease certain hens are "carriers," that is, they have the causal organism permanently present in the ovary. In consequence the eggs laid are infected, and the chick is infected before hatching. This would seem at first to be an example of infectious disease inheritance. It is not such, strictly speaking. The true hereditary mechanism of the cell is not changed. It is true that predisposition to disease may be inherited. We sometimes hear the expression "tuberculosis (or consumption) runs in the family." This means simply that the predisposition to the disease is inherited, for very rarely is an individual tuberculous at birth. Further, the intimate contact of members of a family with each other renders the spread of tuberculosis from one individual to another comparatively easy. It is difficult to tell in all cases just how much the prevalence of a disease in a certain group or family may be due to actual predisposition to the disease on the one hand, or to the unusual opportunities for infection on the other.

Natural Body Barriers to Infection. The *skin* is a comparatively efficient barrier to the entrance of microorganisms. It is continually being renewed from below and scaling off at the surface. Microorganisms frequently penetrate to some depth in the skin, but rarely gain entrance to the living tissues underneath. Very few pathogenic organisms can penetrate through the unbroken skin. Occasionally the organisms pass through the glands or through the hair follicles and produce localized infections such as boils and carbuncles. Just under the skin there are layers of *fascia* or connective tissues which also afford an excellent mechanical barrier to the penetration of microorganisms. These are, moreover, infiltrated with serum, and this serum, as will be shown later, may aid in the destruction of organisms.

The mucous membranes lining the body cavities which communicate with the surface are usually not easily penetrated by organisms. They derive their name from their secretion of mucus, thrown off by certain cells termed *goblet cells*. This mucus catches and retains particles of dust and organisms of all kinds that come in contact with it. This action is particularly marked in the respiratory tract, where the mucus is secreted in considerable quantities by the membrane lining the tracheal and bronchial tubes. Organisms from the air are caught on this moist surface and the mucus is continually being swept up toward the mouth by minute cilia projecting from certain of the cells constituting the so-called ciliated epithelium. Microorganisms also find mechanical barriers to their entrance with the inspired air in the *hairs* which are just inside the nose and serve as filters.

In the mouth large numbers of microorganisms are normally to be found. The *acid secretions* of the gastric glands are sufficiently germicidal so that few bacteria can survive and many bacteria, though by no means all, in ingested food and water are destroyed during passage through the stomach. When the contents of the stomach pass into the intestine, the acid is neutralized by the alkaline pancreatic and other intestinal juices. The reaction becomes therefore more favorable for bacterial growth, but most organisms are inhibited from developing by the fact that the *bile* is antiseptic and will prevent the growth of many species. This is not true of all species, however, as some of the bacteria (as the colon bacillus) normally present in the intestines and some of those which produce intestinal disease and gain entrance to the body through the alimentary tract, as the typhoid bacillus and the dysentery bacillus, are not inhibited in their development by this material. In fact, ox bile is added to the sugar culture medium employed for some of these intestinal bacteria.

Factors Predisposing to Infection. It is a matter of common knowledge that each individual differs markedly at various times in his ability to resist bacterial infection. *Age* is frequently one of the most important predisposing factors to disease. Certain diseases are commonly acquired by children and somewhat more rarely by adults, such as scarlet fever, whooping cough, and measles. On the other hand, there are diseases which are much more common among adults, for example, cancer and diabetes. *Hunger* and *thirst* both decrease body resistance. Exposure to *heat* or chilling of the body surfaces by *cold* may also be predisposing factors to disease. It is found, for example, that if an ordinary barnyard fowl is kept in cold water for some time it loses its natural immunity to tetanus. Excessive *fatigue* also predisposes to disease. This has been many times demonstrated experimentally in the laboratory. If a white rat which is normally immune to the disease anthrax is worked in a treadmill until completely exhausted, then injected with this organism, it will contract the disease and may succumb to it.

The ability of a particular organism to produce disease depends upon several factors. *First* of these is the *virulence* of the organism, that is, its relative pathogenicity (disease-producing or attacking power). It is found in some cases that growing microorganisms under unfavorable conditions or subjecting them to the action of heat or chemicals decreases very considerably their ability to produce disease. It is well known, for example, that in some epidemics of a particular disease, such as diphtheria, the organisms isolated from some infected throats are less virulent than from others. In large part, virulence on the part of an organism is the result of its inheritance. Under a given set of conditions it may be protected from destruction by the cells of the host through the possession of a capsule, it may produce a diffusible toxin, it may excrete spreading factor (hyaluronidase), it may multiply rapidly or slowly. Within the same species of pathogenic organisms there may be great variations in virulence among different strains. These differences may arise as mutations, though in some cases mutation does not seem to be the entire answer.

In epidemic diseases one puzzling phenomenon is sometimes observed. The disease may be rather widespread, but not particularly serious from the standpoint of total morbidity of the population. Then there will arise, frequently from an identifiable center, an infection which is much more serious. This type of widespread mild disease and occasional epidemics of more serious infections are characteristic of such diseases as influenza. A new strain of the causal organism or virus is found to have appeared. What is its origin? There seem to be two or three possible answers. The first is the development of a mutation with addition of factors that make for virulence. Another suggestion has been made by Pontecorvo (1948) that there might occur a recombination of characters or properties of two or more strains of the pathogenic organism. In discussion of the problems of variation in microorganisms it was pointed out that there is some increasing evidence that there may sometimes occur in microorganisms something corresponding to sexual reproduction, with progeny having characters that do not correspond completely to either of the parents. It has been assumed that different strains of organisms may become relatively avirulent in different ways, and when there is a combination of two organisms there might be put together characters that would enhance virulence. A third assumption sometimes made is that there is a gradual increase in virulence by organisms growing in a suitable environment.

A second factor of importance in determining the ability of an organism to produce disease is the size of the dose inoculated, that is, the number of organisms introduced into the body. In the preliminary study of a disease and of the causal relationship of an organism to it, it frequently is found necessary to inoculate large numbers of organisms so as to secure severe or fatal reactions. Such procedure separates those animals completely refractory or immune from those susceptible, but does not differentiate the grades of susceptibility. For studies of relative susceptibility it is found necessary to graduate the doses. The number of organisms required to cause a recognizable infection varies with the different bacteria, the virulence and the resistance of the host. It has been claimed that as small an inoculum as a single cell of the anthrax bacillus may be sufficient to kill a susceptible animal. In many cases much larger inoculations are needed to produce symptoms of disease.

A third factor in determining the infectiveness of an organism is the channel of entrance (the infection atrium). Some kinds of bacteria, such as the tetanus bacillus, may be swallowed with impunity but will produce infection if introduced into a wound.

The ability of an organism to advance through tissues of the body is often determined by its production of histolytic enzymes such as hyaluronidase which dissolve the cementing material (hyaluronate) between cells. In some cases the progress of secondary invaders is facilitated by the activity of some organism producing such an enzyme.

It has already been noted that the ability to produce disease is also dependent upon the relative *resistance* of the particular individual infected. This resistance, or immunity, may be subject to considerable fluctuations in the same individual.

Conversely, differences in resistance on the part of the hosts or other changes in environment may lead to selection and development of strains of microorganisms which show differences in virulence. Gowen (1941) has shown that the frequency with which a true gene mutation occurs is approximately about 1 in 10^8 cell divisions, that is, in a population of about 100,000,000 bacteria it is probable that there would be one representative of a given mutation. Obviously whether or not the mutant multiplies more or less rapidly than the other cells will determine whether it will replace the non-mutating cells. It has been shown, for example, that the *Micrococcus aureus* associated with boils and wound infections is readily destroyed by penicillin in the body, but that resistant mutations are occasionally developed. Such mutants have a decided advantage in comparison with non-resistant organisms, and the mutant may develop more rapidly and replace the original type. Whether the environment within the host or in a culture medium tends to favor the growth of the more or the less virulent strains seems to vary. For example, it was shown by Wellhausen (1937) who worked with the corn wilt bacterium that when mixtures of virulent and relatively less virulent strains were inoculated into susceptible strains of corn, the selection was toward the non-virulent, while injection into resistant corn led to selection of virulent bacterial strains. Similar studies on mice by Zelle (1942) showed selection in favor of virulent strains both in resistant and susceptible animals, while growth in laboratory media selects non-virulent strains. This may explain the fact that certain kinds of pathogenic bacteria are found to lose their virulence when grown on artificial media.

IMMUNOLOGY AND SEROLOGY

A major subdivision of science has been developed about the phenomenon of disease resistance and immunity to infectious disease, the discipline of *immunology*. At the same time there has evolved a whole series of tests and diagnoses based upon the changes and characteristics of the blood serum, and its constituents, and the recognition of *serology*. Some effort should be made to keep these disciplines distinct, even though in some areas the two may overlap. Certain problems in immunology will be first considered, then certain pertinent topics in serology.

Types of Immunity. Immunity to an infectious disease may be *natural* or *acquired*. The former may be subdivided into *racial* or specific immunity and *individual* immunity; the latter into *active* immunity and *passive* immunity.

Natural Immunity. A natural immunity is one that is inherited, it is congenital. Some species of animals are wholly immune to diseases which affect others. Fowls are normally immune to tetanus, the domestic animals never have typhoid fever, and man does not contract hog cholera. Even within a species certain races may be found much less susceptible than are others. The negro is more resistant to malaria than is the white, while the white is apparently more resistant to tuberculosis than is the negro.

The fact that strain differences exist in susceptibility to disease has long been known in plants. An excellent example is to be found in oats. It has been found that more than sixty distinct races of one species of oat rust have been isolated and described, each attacking in a characteristic fashion certain varieties of oats but not the remaining. Conversely, the several varieties of oats each shows toward each rust race some characteristic degree of susceptibility, resistance, or immunity. By suitable genetic techniques of crossing and selections, new oat varieties have been produced that are quite resistant to almost all the rust strains. The methods of crop breeding have been so far developed that with some species combating disease has come to be almost entirely a matter of development of resistant varieties. For example, practically all the oats and wheat grown in the upper Mississippi valley are resistant varieties developed through plant breeding research.

Control of disease in animals through the identification of resistant strains and their use in breeding has not progressed so far as in plants. First, it has been necessary to isolate strains resistant to particular diseases. Before much progress can be made with the major farm animals, the methods need to be worked out with small animals and birds that have a short life cycle and can multiply rapidly. It has been amply demonstrated that strain differences do exist and stocks of relatively susceptible and resistant individuals may be built up for study of such diseases as fowl typhoid and mouse typhoid. For example, by use of a carefully standardized technique of inoculation and selection Schott (1932) found it possible to increase the survival percentage in six successive generations of mice from 18 per cent to 36, 54, 60, 64, 68, and 75 per cent. In Leghorn fowls, Lambert (1932), in four generations increased the percentage of survivors from 10 to 75.

Eventually it is to be hoped that the genetic techniques of breeding for disease resistance in domestic animals and birds may be applied on a scale that will bring improvement in animal production in agriculture.

Acquired Immunity. Acquired immunity is that which develops in an originally susceptible individual. It is not present through inheritance. It may be either *active* or *passive*. The participation of the tissues of the individual concerned in the development of increased resistance is necessary to the development of an active *acquired immunity*. A *passive acquired immunity* is conferred upon an individual by the injection of immune substances produced by another animal or another individual. One who is passively immunized to a disease takes no part in the production or development of this immunity. For example, a person who has diphtheria is after recovery relatively immune to the disease because the body itself has produced immune substances. Such an individual is said to have an active acquired immunity. On the other hand, an individual may be rendered immune for a shorter or longer period by the injection into the body of antitoxins, or other immune substances, developed in the blood of certain animals. This immunity is termed passive, for the body of the individual thus immunized takes no part in the development of the immunity.

Active Acquired Immunity. Active immunity may be acquired in any one of several ways, the method depending very largely upon the type of disease. There are certain diseases which confer upon an individual who has them an active immunity to a recurrence. This is true in such diseases as smallpox, measles, whooping cough, etc. Immunity may also be acquired by *vaccination.* This term is popularly used most commonly in connection with the disease smallpox, but vaccines have been developed which are useful against other diseases of man and animals. By vaccination is meant the injection or inoculation of dead or living organisms into the body of the individual. If living organisms are used, they are attenuated, or rendered inactive or unable to produce a severe type of the disease. In other words, vaccination, in such cases, is the production in an individual of an infection that will run a benign course. Vaccines are used commonly for the prevention of diseases, much more rarely for their cure. Vaccination against typhoid fever, for example, is accomplished by growing typhoid bacilli, suspending them in solution, killing them by heat, then injecting them subcutaneously into the individual to be immunized. The body reacts in much the same manner as though the individual had a mild case of typhoid fever and a considerable degree of immunity is developed. Active immunity may also be acquired by the injection of the products of some kinds of microorganisms. The toxin or poisonous substance produced by the diphtheria bacillus, for example, if injected several times in suitable doses, will cause an individual to become immune to the disease itself.

In some cases active immunity is *localized.* In certain infections the area of body tissue involved may be circumscribed, that is, rather definitely limited, and it is found that tissues that have been thus involved have developed a local resistance to a recurrence of the infection, but that such resistance has not been developed by all parts of the body.

Passive Acquired Immunity. Immunity may be acquired passively only by the injecting of immune substances directly into the individual. These immune substances are in general the products of the active immunization of an animal. For example (as will be later shown), the antitoxin for diphtheria is produced by actively immunizing a horse against the disease, then using the blood serum of the animal for the injection of the human. Passive immunization is of considerable assistance in the cure and prevention of a few diseases, but unfortunately is not applicable to most diseases.

Theories of Immunity. Many theories have been proposed to account for the facts of immunity. The ancients recognized that recovery from certain diseases conferred lasting resistance to a recurrence of the disease. Each significant new theory proposed took into consideration a larger number of factors. Even yet the problem is not satisfactorily solved, not all the questions raised have been answered. Modern theory has become so complex that an adequate summary would take a volume in itself. However, certain of the basic facts that have been demonstrated are of such interest and importance that they should be presented, even though the explanations are perforce inadequate.

The fact that individuals become immune or resistant to certain diseases has been known from the earliest times. It is only within modern times, however, that specific theories have been promulgated to explain this immunity. Four of these theories require brief mention, two of them for their historical importance, and two because they are the ones now held to explain some of the various phenomena.

Exhaustion Theory of Immunity. When bacteria, yeasts, and molds were first cultivated in the laboratory, it was found that growth would not continue indefinitely in a given culture medium, but would cease in the course of a few days or weeks. This cessation was believed to be due to the exhaustion of some nutrient essential to the growth of the organism. A similar explanation was suggested to account for immunity in man and animals. It was believed that certain substances required for the development of the particular disease-producing organism were found in the body and that the organism could grow only as long as these were present. When these substances were exhausted, the individual was thereafter immune because the organism could no longer develop in the body. It was soon discovered, however, that even in the laboratory plenty of food material could be demonstrated in tubes in which all growth had ceased, and it was further noted that the blood serum from an individual immune to certain diseases could be utilized as a culture medium for organisms of the same type, proving conclusively that immunity is *not* due to the complete removal of any particular food substance.

Noxious Retention Theory. It was soon ascertained that microorganisms usually cease growing in a culture medium not because of the exhaustion of food material, but because of the common production by living cells of substances more or less inimical to their growth. It is generally true that the waste products of any cell are injurious to it, and where such tend to accumulate, as in the culture medium of a test tube or flask, they may stop all growth. A similar explanation was applied to immunity. It was urged that particular pathogenic bacteria when growing in the body produce substances harmful to themselves; these substances accumulate, and finally stop growth. This theory was soon found to be quite inadequate. The accumulation of such growth products could not be demonstrated.

Theory of Phagocytosis. Metchnikoff observed that some of the cells of the body, particularly certain types of white blood corpuscles, or *leucocytes,* have the ability to engulf and digest bacteria and foreign particles of many kinds. These cells he termed *phagocytes,* and the phenomenon of ingestion of the bacteria by the cells, *phagocytosis.* He believed immunity to be due to a process of adaptation whereby the phagocytes of the body acquired the capacity of ingesting and destroying pathogenic bacteria of a certain type whenever they gained entrance to the body. This theory in a somewhat more elaborate form and modified in some respects by the theory next to be described has contributed much to concepts generally held at the present time.

Humoral Theory. Ehrlich came to the conclusion that immunity is due to the development in the body fluids, i.e., the body *humors,* of certain specific substances. These substances, so significant in the production of immunity, he called *antibodies.* He found that the injection of many kinds of materials into the body would cause the appearance in the blood, or other body fluids, of substances that would react with them, in some cases neutralize them; in other cases, destroy them; in still other cases, precipitate or dissolve them. Any material injected into the body and proved able to induce the formation of these substances is termed an *antigen.* Any substance formed as a result of the injection of the antigen and reacting with it is termed an *antibody.* For example, the toxin of the diphtheria bacillus when injected into the body in small quantities as an antigen causes the tissues to produce an antibody called *antitoxin* which is capable of neutralizing it. The antibodies produced are of several types: those that can cause *clumping* (*agglutination*) or *precipitation* are called *agglutinins* and *precipitins;* those which can dissolve or destroy cells are termed *cytolysins* or *cytotoxins;* and those which stimulate *phagocytosis* are called *opsonins.* Some have also been described which are chiefly concerned in rendering the body sensitive or highly susceptible. These will be considered in order.

ANTITOXINS AND RELATED ANTIBODIES

Toxins. The word *toxin* is used in several different senses. In some cases it may be essentially a synonym of *poison*. As employed in immunology it usually designates a poisonous substance having certain definite characteristics. The characteristics of a true toxin, i.e., a toxin in this restricted sense, may be formulated as follows.

1. Toxins are poisonous organic substances secreted by living cells of plants or animals. This definition excludes, of course, all poisons of inorganic origin, such as arsenic.

2. Toxins when injected in non-fatal doses into suitable animals cause the animal to produce in its blood or tissues substances called *antitoxins* which will neutralize this poison. In consequence the animal develops some degree of immunity toward the toxin. This excludes from consideration many poisonous substances produced by plants and animals which do not have this characteristic. For example, the poisons opium and strychnine produced by the poppy and nightshade do not cause the body to develop antitoxins and are not toxins in the sense used here.

3. Most toxins are *thermolabile,* that is, they are easily destroyed by heat. Practically all of them are inactivated by brief exposure to the temperature of boiling water, and some of them are destroyed at temperatures much below this. They are also usually inactivated by exposure to light, to oxygen, and to various chemicals.

4. The chemical composition of true toxins has not been accurately determined. It has thus far proved impossible for the chemist even to determine the presence of toxins in solution by the use of chemical means alone. The only method so far devised for their detection and study is that of *animal inoculation*. In short, toxins must be investigated principally by biological or biochemical methods.

5. The introduction of a toxin in the body generally causes no body reaction until some time has elapsed. In other words, toxins are said to show a definite *incubation period*. This is because the toxin after gaining entrance to the body must combine chemically with certain substances for which it has an affinity before damage can be noted.

Toxins are produced by many plants and animals. The venom of the rattlesnake and cobra, the poison of the scorpion and the tarantula, the poisons produced by certain fishes and insects, are animal toxins, or zoo-toxins. Toxins produced by plants (phytotoxins) are also numerous. The castor oil bean and the bark of the locust tree contain powerful toxins known as *ricin* and *robin* respectively. A few bacteria likewise produce toxins, among them the diphtheria bacillus (*Corynebacterium diphtheriae*), the tetanus bacillus (*Clostridium tetani*), and the bacillus causing botulism (*Clostridium botulinum*).

Not all poisons produced by bacteria are true toxins. In some cases they are poisonous products termed *endotoxins* whose nature has not been established definitely. These endotoxins are usually closely bound up with the protoplasm of the cell and are released by its death and autolysis. These endotoxins differ chiefly from the toxins in that when injected into the animal body, they do not cause the production of antitoxins. Endotoxins are of considerable importance in connection with food poisoning.

Preparation of the Bacterial Toxins and Antitoxins. The most commonly used of the antitoxins, the one whose preparation may be used to illustrate the preparation of others, is that specific for diphtheria. For the preparation of the toxin the diphtheria bacillus (*Corynebacterium diphtheriae*) is grown in large flat-bottomed flasks containing a relatively shallow layer of a specially prepared nutrient broth, one which will stimulate maximum production of toxin. The organism is inoculated upon the medium, where it grows as a scum or pellicle over the surface. An adequate oxygen supply is needed to insure the maximum production of toxin, hence the relatively large area of the medium exposed to the air. The flasks are incubated at blood heat until the film of diphtheria bacilli has spread completely over the surface. They are then removed and a little phenol added to destroy the bacteria present. The broth is filtered through a porcelain filter to remove the bodies of the bacteria. The toxin is in the filtrate. The concentration of the toxin must then be determined, for usually no two flasks will be found to contain exactly equal amounts. The method commonly employed for determining the potency of toxin is by injection into animals. Guinea pigs weighing two hundred and fifty grams are used. A series of these animals is injected with graduated amounts of the toxin and the smallest fatal dose accurately determined. This dose is known as the *minimum lethal dose* (abbreviated as M.L.D.). Knowing this, the manufacturer of antitoxin can ascertain how much may be injected with safety into an animal which he intends to use for antitoxin production.

The horse is the animal usually used in preparation of diphtheria antitoxin. Normal healthy horses are secured and every precaution used to ascertain that they are entirely free from any infectious disease. A small quantity of diphtheria toxin is then injected under the skin. Usually the horse will show some reaction, such as fever and refusal to eat. Within a few days the animal becomes normal again and a somewhat larger dose of toxin is injected. These injections are repeated at intervals with increasing doses until very large quantities of toxin are injected at one time, quantities as great as 400 or 500 cubic centimeters sometimes being introduced. Antitoxin appears in the blood, the amount increasing rapidly under this treatment. Immunization is continued for several months, when the blood is harvested by introducing a hollow needle or cannula into the right jugular vein and allowing the blood to flow into a sterile jar. About a liter of blood can be drawn for every 100 pounds weight of the horse without dangerous depletion. The animal is allowed to regain its strength, when injections are renewed and after a time the bleeding repeated. The blood is chilled until it has clotted and the clear straw-colored serum has separated from the fibrin and corpuscles. The serum is pipetted off and constitutes the antitoxic serum used in therapy. Before being used, its concentration or potency is determined by titration against a standard diphtheria toxin. Definite amounts of toxin are mixed with different amounts of antitoxin and injected into guinea pigs. In this manner, the number of *immunity units* is determined. Before being sold, the antitoxic serum is filtered through porcelain to remove any bacteria that may have gained entrance during manipulation. A small amount of preservative is also usually added. The antitoxic serum is placed in vials or closed syringes and is ready for use. Subcutaneous injections in the human are utilized to prevent or cure diphtheria.

Antitoxins may be prepared specific for all of the true bacterial toxins, but relatively few antitoxins other than that specific for diphtheria have come into common use. One of these is the antitoxin produced for the toxin of the tetanus bacillus, the cause of lockjaw. Antitoxins capable of neutralizing the venom of snakes are also prepared commercially. These antitoxins are manufactured in essentially the same manner as that specific for diphtheria, though there are pronounced differences in details. For example, the tetanus bacillus is an anaerobe, and the toxin must therefore be produced in the absence of free oxygen.

The Origin of Antitoxins. It is believed that toxins harm the cells of the body because they combine with certain atom groups of the cell protein and injure the protoplasm. They do not unite indiscriminately with any cell. For example, the toxin of the tetanus bacillus usually unites preferentially with the cells of the nervous system. Apparently these nerve cells are the only ones which contain atom groups or chemical compounds with which the toxin can combine. These atom groups are probably parts of the molecules of the proteins of the protoplasm. They have been termed cell *receptors* (or *side chains,* whence the designation *side chain theory*). It has been urged that these receptors are normally useful in fixing molecules of food to the protoplasm and that their union with a toxin is a diversion from their normal function. It can be demonstrated that after toxins have united with some of these cell receptors, but in quantities insufficient to cause the death of the cell, the latter responds by producing more of this type of cell receptor. This phenomenon is somewhat analogous to production of overgrowths of tissues of the body when injured or irritated. Repeated friction of the skin for example, results in the development of a callus, an overproduction of skin in response to irritation. Apparently the new cell receptors increase in number to such extent that many of them are discharged into the blood stream. These freed cell receptors still retain their ability to unite with molecules of toxin. In short, they constitute the antitoxin molecules. When one of these antitoxin molecules neutralizes a molecule of toxin, the latter is no longer able to unite with a cell and to injure it, somewhat as an acid once neutralized by an alkali can no longer combine with more alkali when this is added.

Cytolysins. A second type of antibody which may be of some significance in the explanation of immunity is the cytotoxin or cytolysin. The injection of cells foreign to a given individual into the blood or tissues of the individual will incite these tissues to the production of substances capable of destroying or dissolving the injected cells. For example, if dead typhoid bacteria are injected into a rabbit repeatedly and in increasing doses and this is followed by injections of living bacteria, the blood of the animal gradually acquires the ability to kill and even partially to dissolve typhoid bacilli. This may be demonstrated by adding the blood serum from this rabbit in sufficient quantities to a suspension of living typhoid bacilli. Cultures made from this suspension at intervals will show that the bacteria are rapidly destroyed. This reaction may be secured not only with bacterial cells but also with cells of other kinds. If the red blood corpuscles of one species of animal are injected into the blood stream of another, the blood serum of the latter acquires the property of dissolving the red blood corpuscles of the first. This general phenomenon of the destruction of cells is termed *cytolysis*, and the substances present in the serum capable of bringing about this destruction are termed cytolysins. A cytolysin which will destroy bacteria is termed a *bacteriolysin*, one which will destroy red blood corpuscles a *hemolysin*, etc.

Bacteriolysins may be of some importance in the development of immunity to certain diseases. The blood serum of a person who has recovered recently from typhoid and who is, therefore, relatively immune to the disease, will be found to destroy typhoid bacteria. In fact, many of the non-pathogenic bacteria, and even some of those which are commonly pathogenic are destroyed by bacteriolysins found normally in the healthy body. When one becomes immune to a disease or develops an active immunity in many cases the amount of this bacteriolysin is considerably increased.

Inasmuch as bacteriolysins can be developed for certain kinds of bacteria by injection of these organisms in increasing doses into suitable animals, it is possible to produce *bacteriolytic* or so-called *antibacterial sera*, in a manner analogous to that used in the production of antitoxin. Usually large animals, such as the goat or the horse, are used for this purpose. The bacteria are grown upon culture media and injected in increasing doses into the animal to be immunized. If this animal is susceptible to the particular disease caused by this microorganism, it is sometimes necessary to begin the injections with dead bacteria and, when a certain degree of resistance or immunity has been developed, to follow this with injections of the living bacteria. The blood is then drawn from the animal and the serum is collected. It is termed an *antibacterial serum*, a *bacteriolytic serum*, or simply an *antiserum*. Unfortunately, bacteriolytic sera have not proved efficient against most diseases. There are a few exceptions to this. Cerebrospinal meningitis in man, for example, is best treated by the use of a specific antiserum. One of the difficulties, perhaps, is the fact that the bacteriolysin developed in the body of one species of animal is not always efficient when introduced into the body of another species.

Bacteriolysins are not as simply constituted as are the antitoxins They may be shown to be made up of two substances; one terme *complement* is present in most normal blood, the other called *amboceptor* is produced as a result of immunization. Further, the complement itself may be shown to be a somewhat complex mixture. Complement is readily destroyed by heat; 56° C. for thirty minutes wil render it inactive. A bacteriolytic serum which is thus heated will lose its power to destroy bacteria. This power is also lost after the serum has been allowed to stand for a time in contact with air, or in a warm place. Such a serum is said to be *inactivated*. This serum can be reactivated, that is, made capable of once more destroying bacteria by the addition of normal serum. In other words, neither normal serum nor the inactivated serum can destroy bacteria, but when the two are mixed, such destruction is possible. The nature of the reaction is, therefore, much more complex than is the case with antitoxins and toxins. It is believed that the amboceptor first unites with the bacterial cell and sensitizes it. The complement is then able to unite with the bacterial cell and destroy it. The complexity of the reaction and the instability of the complement explain in some measure the fact that antisera can be used in the treatment of comparatively few diseases.

Opsonins. Certain body cells termed *phagocytes* are known to be capable of engulfing and destroying foreign bodies such as bacterial cells under certain conditions. Among these phagocytic cells are the *leucocytes,* or white blood corpuscles, and some of the fixed body cells such as those of the endothelium or lining of blood vessels and those of glands such as the lymph nodes. The leucocytes because of ease of study have had most attention though not necessarily the most important. There have been described from normal blood of man five or six different kinds of leucocytes. Some of these are not active in the destruction of microorganisms, that is, in phagocytosis. Important are the forms known as *polymorphonuclear leucocytes*. These are white cells, somewhat larger than red blood corpuscles, containing a nucleus which is very irregular in shape, frequently in the form of a horseshoe or separated into several more or less spherical nuclear segments connected by threads. Certain of the cells possessing round or spherical nuclei are also phagocytic.

These phagocytes are not usually able to engulf and destroy microorganisms without the assistance of certain substances to be found in the blood serum. An experiment such as the following demonstrates this fact. If normal blood is drawn into a solution of sodium citrate or oxalate, coagulation will not occur. The citrated blood may be centrifuged until the blood corpuscles are thrown to the bottom and the serum rises to the top. The blood serum may then be pipetted off and physiological salt solution added to the blood corpuscles. These are then shaken up with salt solution and again centrifuged.

The supernatant liquid is removed and replaced by fresh salt solution. By repeating this process several times the corpuscles may be washed quite free from blood serum. Careful examination of the sediment in the centrifuge tube will show that the white blood corpuscles or leucocytes are most abundant in the surface layer of this sediment and can be removed by means of a pipette. When these are mixed with suitable microorganisms and stained mounts are made from time to time, none or very few of the bacteria will be found within the bodies of the leucocytes. If, however, a suitable blood serum is added to the mixture, microscopic examination will soon show the presence of considerable numbers of bacteria within the leucocytes. The experiment may be varied by mixing the bacteria with the serum, allowing them to stand for a time, and then washing them free from serum. When bacteria treated thus are brought into contact with the washed leucocytes, they are rapidly engulfed. It is evident, therefore, that there is something in the blood serum which so changes the bacteria that they are readily attacked by leucocytes. The bacteria may be said to be rendered *positively chemotactic* to the leucocytes. The substance present in blood serum which brings about this change of bacteria is termed an *opsonin*. This word comes from a Greek word (*opsoneo*) meaning to set a table or prepare a meal; its appropriateness is obvious.

Opsonins which are capable of bringing about phagocytosis of one species of bacterium ordinarily have no effect upon another species, that is, the opsonins resemble other antibodies in being specific. Immunization against a disease may cause the production in the blood serum of a larger amount of opsonin than is present in normal or nonimmune serum. In other words, immunity in some diseases is undoubtedly in part due to the production in the blood serum of substances (opsonins) which enable the phagocytes to destroy the invading pathogenic bacteria. Methods have been evolved for determining the ratio of opsonin in the blood of such an individual to that in the blood of a normal individual. This ratio is termed the *opsonic index*. It is believed, for example, that when the opsonic index of a person that has a certain disease rises above unity, that is, when the opsonin content of the patient's blood rises above that of a normal person, it is to be considered as a favorable symptom, as it indicates that increased phagocytosis is possible.

Vaccines and Vaccination. The use of antisera in production of a passive immunity in many diseases has not proved effective. Protection against infection by use of vaccines has been found useful in many cases. A vaccine is a killed or weakened (attenuated) suspension of organisms introduced into the body for the purpose of causing the development of an active immunity. Injection of virulent microorganisms is usually undesirable but by the use of dead or attenuated cells a reaction or mild attack of the disease may be produced, followed by a development of immunity. Vaccines consisting of dead bacteria are usually termed *bacterins*. Typhoid vaccine is prepared by suspending in physiological salt solution typhoid bacilli grown upon an agar culture and heating until the bacteria are killed. When injected into the body there usually develops some active immunity against typhoid. The vaccine commonly used in smallpox is obtained from the lymph produced on the skin of calves by inoculation with attenuated smallpox virus. The virus of smallpox is known to be present in the lymph in the pustules which develop upon the skin in this disease. This lymph is used for inoculating the calf, and the lymph from the vesicles of the calf is usually mixed with glycerol and sealed hermetically in capillary tubes. This is then used in the production of a mild infection in the human, and as a result of this infection, a considerable degree of immunity to smallpox is developed.

Allergy, Sensitiveness, and Anaphylaxis. It is a matter of common experience that individuals become peculiarly sensitive to the presence of foreign material of many kinds, in some cases to its ingestion, sometimes to its inhalation, sometimes to its injection. Such sensitiveness or hypersensitiveness is called *allergy*.[1] One of the allergic phenomena which has been much studied is the sensitization of the body by the injection of an antigen in such fashion that a subsequent injection properly spaced with reference to the first will evoke more or less serious symptoms of irritation or injury. This is termed the phenomenon of *anaphylaxis*.[2] One of the familiar laboratory demonstrations is to inject a dilute solution of a protein, such as egg albumin, into the body of a guinea pig without repetition of the injection for ten days to three weeks. At the end of this period a second injection will cause severe symptoms of poisoning. Two or three cubic centimeters of diluted egg albumin injected intraperitoneally into such a hypersusceptible animal will kill it within a few minutes. Egg albumin is not to be considered as normally poisonous, but injections made in this manner render the body unusually or abnormally sensitive.

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[1] From the Greek *allos,* another, and *ergon,* work or action.

[2] From the Greek *ana,* against, back, and *phylaxis,* security.

Such an animal is said to be in a state of *anaphylaxis* toward egg albumin as evidenced by the reaction evoked by the second injection. It is interesting to note that this reaction is specific as are the others which have been discussed. If a guinea pig is rendered anaphylactic toward egg albumin, the injection of some other protein, such as blood serum of the horse, will not cause any reaction. On the other hand, the guinea pig may be sensitized to practically any protein and to many of the more complex protein derivatives. If the second injection is made of an amount less than fatal, the animal quickly recovers and is thereafter for a time no longer sensitive, it is desensitized. In spite of much study a wholly adequate explanation of the phenomenon is not at hand. It has been suggested that the tissue cells have developed antibodies which remained fixed to the cells and that when the foreign material comes in contact it is bound in such quantities as to interfere with normal cell functioning.

In man the evidences and symptoms of allergy are numerous and important.[3]

One of the most frequently observed of the allergic phenomena is that of *hay fever*. A person may in some manner become sensitive (allergic) to the compounds present in the pollen of certain plants. Thereafter, whenever this pollen is inhaled, it may cause severe irritation of the mucous membranes of the nose and throat. Similarly, the inhalation of dust from various sources may be the cause of *asthma*. The sensitiveness of an individual to a particular type of protein or other foreign material may be tested by the injection of a small quantity of this material just under the skin or by rubbing it vigorously into the skin. The tissues of a person who is sensitive will become inflamed and somewhat swollen, while those of a normal person, that is, one who is not in a condition of allergy, will exhibit no marked reaction.

Similarly, individuals may sometimes become sensitized to the proteins or growth products of certain bacteria. This occurs most frequently in some of the chronic diseases. It is believed that the phenomenon of anaphylaxis explains certain rather obscure body reactions in such diseases. The common method of diagnosing tuberculosis in animals (particularly in cattle and to some degree in man as well) is by the injection of a small quantity of a killed culture of *Mycobacterium tuberculosis* subcutaneously into the suspected individual. An animal that has no tubercle bacilli growing in the body will show no change, but one that is tuberculous will show within a few hours a definite reaction in which the temperature rises and in the course of a day or two comes back to the normal. It is probable that the explanation of this reaction is to be sought in anaphylaxis. The presence of the tubercle bacilli in the body has sensitized the body to the products of this organism and the injection of the dead tubercle bacilli, or *tuberculin* as the material is called, causes, on the part of the body, a reaction of the same general nature as that which is produced in the guinea pig by the second injection of the protein, or by the injection of the suitable pollen into one who has hay fever.

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[3] Antigens which cause the development of allergy are sometimes termed *allergens* and the antibodies produced in the tissues *reagins*.

This method of diagnosis has also been used in certain other diseases, as in glanders of the horse.

Serology. We have thus far been considering those factors and agencies which seem to be significant in disease resistance and immunity. In man and animals the blood and its serum have been found to contain various antibodies significant in immunology. As studies progressed in this field it became evident that various other antibodies might be demonstrated, perhaps not significant in the development or explanation of immunity, but of real use in disease diagnosis, and in differentiation and identification of microorganisms, of other cells, and of proteins. Among these antibodies are the agglutinins and precipitins. The complement fixation test and the hemolytic tests have also proved helpful.

Agglutination and Agglutinins. It was discovered by Gruber and by Widal that when a little of the blood serum from an individual suffering from typhoid fever is introduced into a broth culture, or suspension, of typhoid bacilli, these organisms rapidly clump together and settle to the bottom of the tube. When the process is watched under the microscope, the bacteria are observed gradually to lose their power of motility and to gather in clusters or clumps. These bacteria are not necessarily destroyed by this process, for when placed upon the surface of suitable culture medium, they will be found to develop normally. Similar phenomena have been noted in the blood of animals and man affected with diseases other than typhoid fever. Blood serum from a person that has dysentery, for example, will clump or agglutinate the dysentery bacilli, that from one having undulant fever will clump the bacilli characteristic of that disease. The reaction, therefore, is said to be *specific*.

Much use is made of agglutinins in the diagnosis of disease. When a physician wishes to determine whether or not a patient has typhoid fever, he is greatly aided in his diagnosis by the results obtained from the *agglutination* or *Widal* test. He draws a drop or two of blood from the tip of the finger or the lobe of the ear, separates the serum, and mixes it in dilutions of 1–20 and 1–40 with a suspension of typhoid bacilli. The reaction may be observed under the microscope, and if the characteristic clumping occurs in the course of half an hour, the diagnosis is positive for typhoid fever. The test may also be carried out using larger quantities in tubes with observations of the results directly without use of the microscope. This agglutination test is useful in diagnosis of many diseases, but cannot be used for all. In some cases specific agglutinins are not produced in the blood, and in other cases the difficulty of getting a homogeneous suspension of the bacteria renders the method impracticable.

The substances developed in the blood capable of causing agglutination are called *agglutinins*. They may be induced in laboratory animals by the systematic injection of killed or living cultures of the bacteria for which agglutinins are desired.

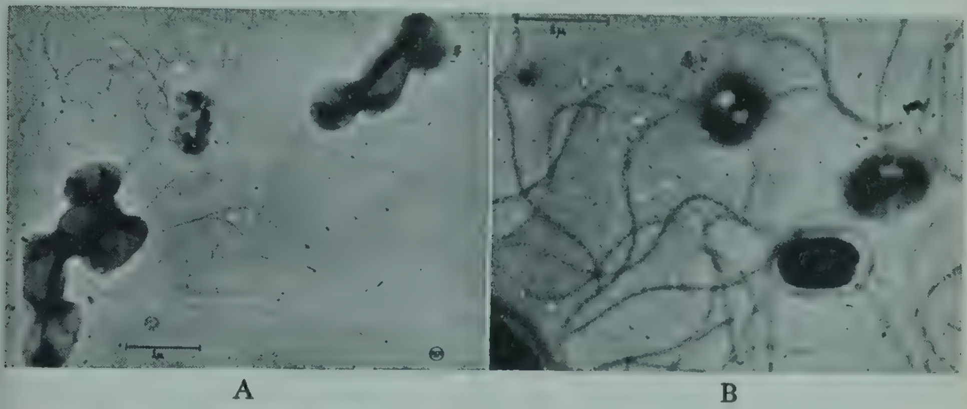
Just what agglutinins are and how they bring about clumping is a problem which can be answered only by a study of certain facts which have been developed in the field of colloidal chemistry. The flocculation of colloidal suspensions has received intensive study in recent years.

A suspension of bacterial cells in many respects behaves as does a true colloidal suspension. One of the factors which tends to keep particles in suspension is the electric charge which they carry. Bacterial cells in suspension may be shown usually to carry such charge, and inasmuch as all are charged alike, they tend to repel each other. On the other hand, the bacterial cells have some tendency to stick to each other, or cohere. Whether or not they clump is determined in large part by the relative strength of the repulsive force on the one hand and the cohesiveness of the cells on the other. Many substances are known which when added to a suspension may alter the charge on the particles, or alter the cohesiveness. Among such substances are certain salts.

Apparently agglutinins unite with the bacterial cell and change its sensitiveness to precipitation by salts. If agglutinin and bacteria (both salt-free) are mixed, agglutination does not occur until some salt is added.

It seems that the agglutinin in part coats the cell and changes the cell suspension from one which is *hydrophilic* to one which is *hydrophobic,* that is from one which tends to remain in suspension in water to one which precipitates readily. Agglutinins may be quite as effective in causing agglutination in suspensions of killed cells as in one in which the cells are alive.

Agglutinins may constitute a part of the general mechanism of immunity, but the exact function they perform, if any, is not clear. Their usefulness in the laboratory is, however, very definite. They are utilized in the following ways.



A B

Fig. 29–1. A. Electronograph of cells of *Salmonella typhosa* showing the numerous slender peritrichous flagella. These cells have not been exposed to serum. (Courtesy of S. Mudd, K. Polevitzky, and T. F. Anderson and *Archives of Pathology.*) B. Electronograph of cells of *Salmonella typhosa.* Note that the magnification is slightly greater than in A. The cells have been exposed to an antiserum containing antibodies specific for this organism. Note that the flagella appear much thicker owing to the deposition of the specific antibody globulin on the surfaces. There is evidence also of the precipitation of the serum proteins on the surfaces of the cells. Note that the protoplasm has shrunken away from the cell wall in some cases. (Courtesy of S. Mudd and T. F. Anderson and the *Journal of the American Medical Association.*)

1. *Disease Diagnosis and Differentiation.* It has been found possible to diagnose some diseases, such as typhoid, as noted above. It is also practicable to differentiate cases of paratyphoid from those of typhoid which are clinically somewhat similar.

2. *Differentiation of Bacteria.* It is possible to identify bacteria by use of appropriate homologous sera. For example, if one has isolated a number of pure cultures of bacteria from contaminated water, it is comparatively easy to determine which are cultures of typhoid bacilli

by use of a serum known to contain agglutinins specific for typhoid bacteria.

However, the situation is much more complex than has been thus far pictured. Bacteria generally contain or produce more than one kind of antigen which can provoke agglutinin production in the body. In the case of a motile organism it may be shown that agglutinins may be produced that are specific for the flagella, the *flagellar agglutinins*, and others that are specific for the cell itself, the *somatic agglutinins*.[4] Furthermore, the capsular layer of the bacterial cell may have different antigens from those of the protoplasm of the cell. Some of the antigens are readily destroyed by heat, they are *thermolabile;* others are relatively heat resistant, they are *thermostable*. To make the situation still more complicated, the antigens present in one kind (species or variety) of organism may in part be the same as those in another. The problem of how to dissociate these antigens and their corresponding agglutinins has led to a great concentration of effort to bring order out of a very complicated situation and to make the greatest practicable use of the findings in the differentiation of organisms and the diagnosis of diseases.

It was found that if two organisms A and B have some common antigens, antisera prepared by injections of A into a suitable animal will agglutinate both A and B. Likewise antisera prepared by use of B will agglutinate A. That the agglutinins in the two antisera are not all identical may be shown by *agglutinin absorption*. When an excess of bacteria is added to the corresponding (*homologous*) antiserum, the agglutinins are adsorbed quite completely to the bacterial cells. Bacterium A added to antiserum A will remove the agglutinins. If the agglutinated bacteria are removed from the serum, and a new suspension of A added, no agglutination will take place. When A bacteria are suspended in B antiserum, the cells will be agglutinated and may be removed. The B antiserum will be found to have lost its ability to agglutinate organism A. But when B cells are added to this antiserum they will be agglutinated. It may also be shown that antiserum A will agglutinate B cells, and when the latter are removed agglutinate A cells but not B cells. Clearly in this case both A and B cells possess at least two kinds of antigens, and the corresponding antisera two kinds of agglutinins, there are agglutinins specific for A but not B in antiserum A, specific for B but not A in antiserum B.

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[4] From the Greek *soma*, meaning body.

and each antiserum contains an agglutinin which will agglutinate both A and B.

By use of a series of related organisms, it may be shown that each organism has several to many antigens and the corresponding antisera correspondingly several to many agglutinins. Then, too, it is possible to differentiate agglutinins for somatic and flagellar, for thermostable and thermolabile antigens.

Clearly, then each kind of organism is a mosaic of different antigens. Some of the antigens of the mosaic of organism A may be characteristic, others may be common with those of organism B, still others common to A and C but not to B, some common to B and C but not to A, and some common to all three. In certain groups or genera of bacteria extensive studies have been made to determine the mosaic pattern of different strains. More than 150 such patterns have been determined in the genus *Salmonella* alone. A method (to be noted later in discussion of *Salmonella*) of shorthand indication of the components of the mosaic has been developed and distinctive names given to each of the different strains described. For example, the formula for *Salmonella enteritidis* is [I], IX, XII—g.m. White and Kauffman were the original proponents of this method of differentiation which has found rather wide acceptance.

Those who advocate this method of differentiation and identification have sometimes tended in their writings to suggest that this use of antigens is superior to other methods. Antigens are, after all, definite chemical compounds and the serological method, while possibly convenient, is a priori no better than any other method of determining characteristic chemical compounds in cells. Mutations change antigenic patterns just as they change fermentive or pigment or morphology patterns.

Precipitation and Precipitins. We have noted above that the injection of suspensions of cells of a microorganism into an animal will cause the animal to produce agglutinins specific for those cells. It has also been determined that the injection of various complex compounds, particularly proteins, in *solution* will cause the formation in the animal body of similar substances which will *precipitate* these proteins. If, for example, a dilute solution of egg albumin is injected at intervals of a few days into a rabbit, and a few drops of the blood of that rabbit then drawn and the serum is added to a solution of the albumin, a precipitate will be formed. The phenomenon differs from that of agglutination in that in the latter the bacteria are in suspension, in the former, a protein is in solution. The two phenomena are closely related, for both bacteria and proteins form colloidal suspensions, the primary difference being merely in particle size. The antibody developed capable of causing precipitation is termed a *precipitin.*

This precipitation phenomenon is utilized in several ways. The reaction is very specific; that is, the blood serum of an animal immunized against one kind of antigen will cause precipitation of that particular type only. The protein of muscle of two different species is so similar chemically that it would be difficult if not impossible for the chemist to detect a difference, but by use of the precipitation reaction differences may be found readily. Practical uses of precipitation are numerous. For example, the sale of horseflesh for human food in some countries is permitted by law provided that it is not sold as beef. There are certain chemical differences between the fat of horseflesh and that of beef, and in the fresh tissues there are some differences in the amount of glycogen present, but these differences are not readily detected, and chemical analyses are not always reliable. Antisera containing precipitins specific for horsemeat are prepared from blood of an animal, usually a rabbit, previously injected with an extract of horsemeat. The meat being tested is minced, extracted with water, and the juice expressed. Various dilutions of the specific antisera are then added to the samples of the extract. If the meat is horseflesh, a precipitate will be produced, otherwise not. In the latter case, a test may be made using the blood serum from the animal immunized against beef, and the precipitate should develop. This method may also be used to differentiate the various meats that are used in the preparation of sausage. An extract made from sausage should be precipitated by the serum from animals immunized against each of the kinds of meats used in that sausage. Such determinations are difficult if not impossible by chemical or microscopic means.

The origin of blood stains may be determined by use of the precipitation reaction. It is sometimes necessary in legal procedure to determine whether a particular blood stain is of human or animal origin. Rabbits or other laboratory animals are systematically immunized against human blood by repeated injections at intervals of a few days. The blood serum from such animals is then tested by mixing with serum from human blood, when a precipitate should be produced. The blood stain in question may then be tested by dissolving the serum in physiological salt solution, adding some of the serum from the immunized animal, when a precipitate will form if the blood is of human origin but not if of other origin.

Complement Fixation and Diagnosis. We have already discussed the production of bacteriolysins and the role of complement in the blood. Cytolysins of many types have been produced and studied. Several techniques useful in diagnosis have been developed, in a sense as byproducts of these studies. Much of the work has been done with *hemolysin,* antibodies capable of *lysing* or dissolving red blood cells. This is in part because of the ease with which the destruction of red blood corpuscles may be observed. Experimental work may be carried on with red blood corpuscles from any one of several species of animals. Probably those from the sheep are most commonly used. For production of a hemolytic antiserum the rabbit is most frequently employed. The blood from the sheep is either defibrinated or mixed with a solution of sodium citrate to prevent coagulation. It is then centrifugated and the clear supernatant serum removed from the sedimented red blood cells. The latter are suspended in physiological salt solution and again centrifugated. This washing process may be repeated several times. Eventually a suspension of red blood corpuscles in physiological salt solution is secured relatively free from serum. These are injected repeatedly at suitable intervals into a rabbit to produce development of hemolysins in its blood. The animal is bled and the blood serum is secured. A drop of this blood serum added to a suspension of the red blood cells of the sheep will cause the latter to go more or less completely into solution. Any cells which are not dissolved soon settle to the bottom. The supernatant liquid is more or less strongly tinged with red, the depth of color observed depending upon the number of cells dissolved.

The development of bacteriolysins (or specific amboceptor) in the blood of an individual suffering from certain chronic diseases may be utilized as a means of diagnosing the disease. An examination of the blood for the specific amboceptor may then constitute a satisfactory method for diagnosis. This is most easily carried out by the technique described below. The general method has been employed particularly for the recognition of glanders in the horse and a modification of it (called the Wassermann test) for the diagnosis of syphilis in man. The test is usually called the complement fixation test for the diagnosis of disease. This test is carried out by use of the following materials.

1. *Bacterial Antigen.* This is a suspension of bacteria specific for

the disease. If the disease being diagnosed is glanders, for example, this would be a suspension of glanders bacilli.

2. *Serum from the Patient.* If this is from a horse having glanders it should contain the amboceptor specific for the glanders bacillus. It would also contain complement. The latter is destroyed by heating the serum to 56° C. for 30 minutes.

3. *Blood Serum from the Guinea Pig.* This blood serum may come from any normal animal. It contains complement but no amboceptor.

The test is initiated by mixing suitable proportions of Nos. 1, 2, and 3. If the disease is present, the amboceptor will unite with the sensitized bacteria, that is, the complement will be *fixed* by the bacteria. If the horse is not diseased there will be no amboceptor present, and the complement will remain in solution. It is evident that a suitable reagent for detecting whether or not the complement has remained in solution is required. Such a reagent is to be found in sensitized red blood corpuscles.

4. *Sheep Red Blood Corpuscles.* These should be washed and suspended in physiological salt solution.

5. *Blood Serum from a Rabbit Which Has Been Immunized by Repeated Injections of the Red Blood Cells of the Sheep.* This is heated to 56° C. for 60 minutes to destroy the complement which is present.

Numbers 4 and 5 are mixed in suitable proportions. The amboceptor specific for the red blood cells unites with them. This mixture containing the sensitized blood cells is next added to the mixture of Nos. 1, 2, and 3 noted above. It is evident that if there is any complement remaining in solution, the red blood corpuscles will be dissolved. If, on the other hand, the complement has disappeared from solution because of the presence of the glanders amboceptor, the red blood cells will not be dissolved. In reading this test, positive hemolysis indicates the disease to be absent; no hemolysis indicates the presence of the disease or of the specific amboceptor in the horse.

CHAPTER 30

Disease-Producing Microorganisms: Their Separation into Groups

The microorganisms that produce disease to be considered in this section may be grouped in one of two ways. It is possible to place primary emphasis upon the type of disease produced, that is, classify the organisms on the basis of the pathological syndrome as it appears in the host. The second method is to classify them on the basis of their apparent relationships as organisms. This means that the outline in the main should follow the classification of the microorganisms as previously developed. In such a bacteriological classification the closely related organisms are grouped together. For example, we may speak of the intestinal group of organisms which includes all those bacteria which have certain common morphological and cultural characters and habitats, but differ among themselves in minor ways. A *pathological classification* disregards the relationships of the *organisms* and classifies together those *diseases* which resemble each other. For example, all diseases in which toxins are produced and the organisms remain localized (toxemias) may be classified together regardless of the organisms causing these various infections. Or again, all organisms capable of producing nodules or tubercles in the tissues are sometimes grouped together with the *Mycobacterium tuberculosis* under a single heading, in spite of the fact that the organisms themselves are not closely related, and solely on the basis of lesions produced. For our present purpose the bacteriological classification will prove the more satisfactory and will be followed here.

Four principal groups of organisms will be considered, the *pathogenic bacteria,* the *viruses and phages,* the *yeasts and fungi,* and the *protozoa.*

Inasmuch as we are here interested principally in the organisms capable of producing disease in man and animals, and having in consequence considerable economic importance, many of the subgroups commonly discussed under the heading of pathogenic bacteria may be eliminated. A chapter is also devoted to bacteria pathogenic for plants. The groups of pathogenic bacteria of general significance may be differentiated by use of the following key. Keys to other genera of bacteria, some of which may contain pathogenic species, may be consulted in the Appendix.

The fifteen groups of pathogenic bacteria here listed are not inclusive. There are several others containing organisms and diseases less common or well known which are omitted.

Key to Principal Groups of Pathogenic Bacteria

a. Organisms not obligate intracellular parasites.
b. Cells spherical. 1. Coccus group. Genera *Streptococcus, Micrococcus, Diplococcus, Neisseria.*
2b. Cells elongate, not spherical.
c. Motility if present due to flagella; cells not creeping or flexuous.
d. Not producing elongated filaments and mycelium.
e. Cells not acid-fast.
f. Cells not producing endospores.
g. Cells gram-positive.
2. Diphtheria group. Genus *Corynebacterium.*
2g. Cells gram-negative.
h. Not requiring special growth factors or hemoglobin.
i. Polar staining conspicuous.
3. Plague group. Genus *Pasteurella.*
2i. No polar staining.
j. Infecting animals or man, not plants.
k. Flagella if present not polar.
l.
4. Intestinal group. The enterobacteria. Genera *Escherichia, Aerobacter, Proteus, Eberthella, Salmonella, Shigella.*
2l.
5. Undulant fever-abortion group. Genus *Brucella.*
2k. Flagella polar.
6. Group of pseudomonads and vibrios. Genera *Pseudomonas, Vibrio.*
2j. Infecting higher plants.
7. Plant pathogen group. Genera *Pseudomonas, Xanthomonas, Erwinia.*
2h. Hemophilic, requiring hemoglobin or special growth factors.
8. Hemophilus group. Genera *Hemophilus, Dialister.*
2f. Rods producing endospores.
9. Spore-producing group. Genera *Bacillus, Clostridium.*

2e. Cells acid-fast
        10. Acid-fast group. *Mycobacterium.*
2d. Cells elongating to filaments and mycelium.
        11. Ray bacteria group. Genera *Actinomyces, Nocardia, Streptomyces.*
2c. Motility not due to flagella.
d. Cells flexuous.
        12. Spirochete group. Genera *Treponema, Borrelia.*
2d. Cells move by creeping.
        13. Myxobacter group. Genus *Chondrococcus.*
2a. Organisms obligate intracellular parasites.
        14. The Rickettsias. Genus *Rickettsia.*

CHAPTER 31

The Pathogenic Cocci

THE GENERA MICROCOCCUS, STREPTOCOCCUS, DIPLOCOCCUS, AND NEISSERIA

The pathogenic cocci with which we shall be concerned belong to four of the genera of spherical or oval bacteria. These four genera may be differentiated microscopically as follows:

Genera of Pathogenic Cocci

a. Cells gram-positive.	
b. Cells in irregular masses, not in pairs or chains.	1. *Micrococcus* (*Staphylococcus*)
2b. Cells not in irregular masses.	
c. Cells typically in chains.	2. *Streptococcus*
2c. Cells typically in pairs.	3. *Diplococcus*
2a. Cells gram-negative.	4. *Neisseria*

The organisms belonging to these genera from the standpoint of disease production may be placed in two subgroups, the members of the genera *Micrococcus* and *Streptococcus* as the causes of nonspecific infections, and the genera *Diplococcus* and *Neisseria*, whose species produce specific diseases.

THE SUBGROUP OF NONSPECIFIC PYOGENIC COCCI.

The organisms belonging to this subgroup are associated with several types of infections, usually not specific. They are found as the cause of pus production in wounds, boils, carbuncles, abscesses, and in inflammations of the skin, mucous membranes, and underlying tissues, such as erysipelas and tonsillitis, in inflammation of glands as the udder (mastitis). Occasionally they gain entrance to the circulation and may produce septicemia by multiplication in the blood stream itself. Some, when they have grown in certain foods, may produce one type of food poisoning.

Many species of bacteria have been described belonging to these genera. Even yet there is not complete agreement as to number of species and as to nomenclature. The most important of these are *Micrococcus aureus* with the closely related *M. albus,* and *Streptococcus pyogenes.* These are all cocci, do not produce spores, are nonmotile, gram-positive, aerobic, and facultative anaerobic, and grow upon most of the common laboratory media. The genera are differentiated in that the cells of *Micrococcus* are irregularly massed or grouped while those of *Streptococcus* occur in chains. *Micrococcus albus* is differentiated from *M. aureus* in that the latter usually produces a golden yellow pigment when grown upon laboratory media, and the former is white.

These organisms are said to be *pyogenic,*[1] and the process of pus production induced is termed *suppuration.* When these organisms gain entrance to the body (this is true also of other organisms), they produce an inflammation. They begin to destroy and to some extent to disintegrate the tissues with which they are in contact. Substances more or less poisonous in nature are produced, possibly in part the results of synthetic action of the organism, but probably in greater part the products of the breakdown of the tissue cells. The blood vessels in the surrounding tissues become engorged with blood. Blood serum leaves the vessels and infiltrates these tissues, usually causing swelling. The white blood corpuscles also leave the capillaries in large numbers and pass out among the tissue cells. In the immediate vicinity of the infected tissues, they form an almost solid mass. The bacteria are thus surrounded by what may be termed a phagocytic wall which quite effectually prevents their spread, provided opsonins are present in sufficient quantities to enable the leucocytes to engulf and destroy the organisms. These leucocytes invade the diseased tissues and eventually destroy the bacteria present. This is not always easily accomplished, as many of the leucocytes themselves are destroyed by microorganisms. The pus found in such lesions is composed of a mixture of blood serum with white blood corpuscles or leucocytes, bacteria, and disintegrated tissue.

It is only since the last decade of the nineteenth century that pus production has come to be looked upon as an abnormal process. It was formerly supposed to be a part of the natural process of healing of wounds. Boils and abscesses were supposed to be efforts on the part of the body to rid itself of poisonous substances found in the blood. Something of this idea still persists, for one not infrequently hears the expression that a boil or series of boils is useful in that it tends to purify the blood. When it was determined, however, that microorganisms are in most cases the cause of suppuration, efforts were made by the use of *antiseptics* to prevent their development in wounds. This led to a great increase of efficiency in surgical operations. Another great step in advance was the introduction of what may be termed *aseptic surgery*, in which every effort is used to prevent the introduction of microorganisms into wounds by the use of sterile instruments, by careful disinfection of the skin, etc. It is practically impossible wholly to prevent the introduction of organisms, inasmuch as some are usually present in the deeper layers of the skin. The natural body immunity will ordinarily dispose of these, provided other organisms are not introduced in great numbers.

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[1] From Greek *pyon,* pus, and *gen,* to produce.

Micrococcus aureus and Micrococcus albus

Synonyms. *Micrococcus pyogenes aureus, Staphylococcus aureus. Staphylococcus pyogenes aureus.*

Synonyms. *Micrococcus pyogenes albus, Staphylococcus albus. Staphylococcus pyogenes albus.*

The genus *Micrococcus* is often divided into two genera, *Micrococcus* in the narrower sense to include the common saprophytic forms, and *Staphylococcus* for the species which are parasitic and which infect man and animals. It is difficult to find good differences between the two genera, hence the tendency to recognize but one, the older genus *Micrococcus.*

The first definite report of the presence of microorganisms in pus is that given by Ogston in 1881. Rosenbach in 1884 grew them in pure culture on artificial media and differentiated the two species under consideration. These microorganisms are present quite commonly upon the skin and hair of man and animals. They are also usually found in the mouth and not infrequently in the intestines. They are not uncommon in dust.

Morphology and Culture. These organisms are spherical; occasionally where they occur as diplococci they may be somewhat flattened at the point of contact. They are commonly designated *staphylococci,*[2] as the cells ordinarily occur in irregular masses. The cells are somewhat less than 1 μ in diameter, usually between 0.7 μ and

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[2] From the Greek *staphyle*, a bunch of grapes, and *coccus*, a berry.

0.9 μ. They are easily stained by the common laboratory aniline dyes and are gram-positive.

They grow well upon the ordinary laboratory media. Pure cultures frequently may be obtained by smearing a small drop of pus over the surface of an agar slant by means of a platinum wire or loop. Often, however, they do not occur in pure cultures, and it is necessary to plate in order to get the separate distinct colonies. Milk is usually curdled, becomes slightly acid, and eventually the curd is digested. Colonies upon gelatin plates or stab cultures in gelatin show rapid liquefaction. The principal point of difference between these organisms is that the *Micrococcus aureus,* upon most culture media containing carbohydrate and in presence of oxygen, produces a golden yellow pigment, whereas *M. albus* is white. These organisms are quite resistant to drying. This accounts for the presence of living organisms of these species in the air. The temperature optimum is 28°–37° C. They are easily destroyed by disinfectants; 60° C. for one-half hour is usually sufficient to destroy all cells; occasionally, however, strains are found that require heating to a higher temperature.

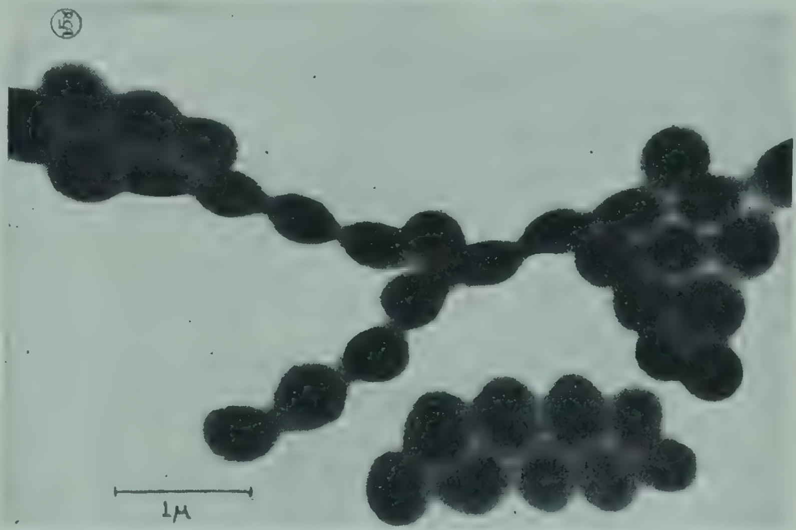

Fig. 31–1. *Micrococcus aureus.* Electronograph. The irregular massing of the cells is clearly shown, as also the cell walls. (Courtesy of S. Mudd and T. F. Anderson and the *Journal of the American Medical Association.*)

Disease Production. Various poisonous substances have been described from staphylococci. Most pathogenic strains form hemolysins easily demonstrated by growing on a medium containing red blood cells. Some produce an enterotoxin frequently associated with food poisoning. Broth culture filtrates injected intradermally produce necrosis (cell death) of the adjacent skin. Such a filtrate is lethal when injected intravenously into the rabbit. These organisms are associated with a great variety of pyogenic infections, abscesses, carbuncles, boils, furuncles, and acne. Occasionally they may gain entrance to the blood stream and produce septicemia or pyemia. In some cases, also, the organisms are carried by the blood stream to the bone marrow and cause inflammation (osteomyelitis). Occasionally, too, they may settle down upon the lining membrane of the heart, or the heart valves, and cause one type of heart disease (endocarditis). Inflammation of the udder in cows (mastitis) is also occasionally found to be caused by organisms of this type. It sometimes happens that an individual who is suffering from some chronic disease, such as tuberculosis, may have a secondary infection produced by these organisms which may prove to be the immediate cause of death.

The staphylococci constitute one of the three groups of bacteria commonly associated with food poisoning. When they have an opportunity to grow in suitable foods at room temperatures or above they rapidly produce a toxin, termed an *enterotoxin*, which is somewhat heat-resistant, and when ingested with food produces poisoning in from two to four hours. While the symptoms of poisoning are severe, the mortality is low, practically negligible, and recovery is prompt. In general, foods which favor the rapid development of staphylococci and of the enterotoxin have starch used as a thickening, such as filling of cream puffs, eclairs and certain types of custards. The food poisoning staphylococci are usually hemolytic and coagulase positive. The presence of coagulase may be demonstrated by placing 0.5 milliliter of a 1–3 dilution of human blood plasma and adding a loopful of the organism to be tested taken from a young agar culture. In the presence of a pathogenic strain, the plasma if kept at 37° C. will usually coagulate within an hour. The enterotoxin produced in the laboratory has been shown to be able to produce the characteristic symptoms in man and certain monkeys.

Immunity. Bacterins (killed cultures) of staphylococci have been extensively tested in the past in cases of chronic suppuration. They have been most successful where they are prepared from cultures taken from the particular individual to be treated, that is, by autogenous vaccines. The development of the sulfa drugs and particularly the discovery of penicillin and other antibiotics has much simplified the treatment of infections caused by staphylococci, for these agents have almost warranted their designation as specifics. However, the staphylococci sometimes produce mutants which are resistant to the action of penicillin. When this occurs, cure of an infection may prove more difficult. Certain strains of *Micrococcus aureus* are usually employed in determining the potency of penicillin.

Streptococcus

The genus *Streptococcus* contains many species. Twenty-four species are recognized in Bergey's *Manual,* and the names of more than a hundred others are listed but not recognized. Some are pathogenic or parasitic, others are saprophytic. Some of the species are obligate anaerobes. Of the saprophytic forms, *Streptococcus lactis* of milk has been discussed in Chapter 24. We shall consider here only the parasitic and pathogenic species which are facultative anaerobes.

The several species have been separated in some cases on the basis of physiologic reactions, in others by the use of serologic tests. Here discussion will be limited largely to *Streptococcus pyogenes* most commonly associated with disease in man, with brief mention only of the closely related *S. equi* producing the disease strangles in the horse, *S. agalactiae* of mastitis (udder inflammation in cows), and *S. mitis* of human endocarditis.

Lancefield made valuable contributions to a grouping of the pathogenic and parasitic β hemolytic streptococci by means of precipitin tests. Each of her several groups can be differentiated by the possession of a characteristic polysaccharide haptene, the groups being further subdivided into serotypes by possession of protein-like antigens. She distinguished a group A in which the organisms are primarily pathogenic for man, group B usually associated with mastitis in cattle, and group C from infections in lower animals.

The streptococci producing the green coloration on blood agar are not readily differentiated by precipitation tests. They are common in the alimentary tract in man and may produce endocarditis. Lancefield includes them in her group D.

The classification of species of the sixth edition of the Bergey *Manual* is perhaps the best attempt to align the various groups into species. The following key is an adaptation.

Key to Species of Pathogenic Streptococci, Exclusive of the Anaerobes

a. No growth at 10° C. Not tolerant of 0.1 per cent methylene blue, or 6.5 per cent NaCl, or pH = 9.6.
  b. No growth at 45° C. Generally do not curdle litmus milk. Litmus reduced slowly if at all. *Generally producing β hemolysis.* (Pyogenic group).
    c. Sodium hippurate not hydrolyzed.
      d. Acid produced from trehalose.
        e. Acid from lactose.
          f. Lancefield group A. *Streptococcus pyogenes*
          2f. Lancefield group C. *S. equisimilis*
        2e. No acid from lactose. (Lancefield group C.) *S. equisimilis*
      2d. No acid from trehalose.
        e. Acid from sorbitol. (Lancefield group C.) *S. zooepidemicus*
        2e. No acid from sorbitol. (Lancefield group C.) *S. equi*
    2c. Sodium hippurate hydrolyzed. (Lancefield group B.) *S. agalactiae*
  2b. Growth at 45° C. (usually). Reduces litmus after curdling milk. Not β hemolytic. (Viridans group.)
    c. Acid from lactose.
      d. No growth at 50° C. Greening in blood agar usually. Growth in 2 per cent NaCl.
        e. Do not survive 60° C. thirty minutes. Not bile tolerant.
          f. Colonies on sucrose and raffinose media mucoid. *S. salivarius*
          2f. Colonies on sucrose and raffinose media not mucoid. *S. mitis*
        2e. Survives 60° C. thirty minutes. Bile tolerant. *S. bovis*
      2d. Growth at 50° C. No action on blood. No growth in 2 per cent NaCl. *S. thermophilus*
    2c. No acid from lactose. Bile tolerant. *S. equinus*
2a. Growth at 10° C. *and* at 45° C. Usually reduce litmus milk before curdling. Tolerate 0.1 per cent methylene blue, 6.5 per cent NaCl or pH = 9.6. (Lancefield group D.)
  b. Not β hemolytic.
    c. Gelatin not liquefied. *S. faecalis*
    2c. Gelatin liquefied. *S. liquefaciens*
  2b. Producing β (true) hemolysis.
    c. Acid from mannitol and sorbitol. *S. zymogenes*
    2c. No acid from mannitol and sorbitol. *S. durans*

Streptococcus pyogenes and Related Species

Synonyms. *Streptococcus erysipelatis, S. scarlatinae.*

This organism was isolated and described at about the same time as were the staphylococci. It was first believed that distinction was to be drawn between the organisms isolated from erysipelas and those from other types of inflammation, but it was soon decided that these did not differ sufficiently to justify their separation into distinct species.

All the streptococci producing disease in man or present upon the skin or in the body cavities will be considered together. The organisms do not grow as well upon artificial media nor adapt themselves as readily to conditions in nature outside of the body as do the staphylococci. They are not uncommon upon the surface of the normal healthy skin, are quite constantly present in the mouth and throat, and may easily be isolated from the feces.

Morphology and Culture. *Streptococcus pyogenes* is a coccus occurring in chains, sometimes short and consisting of three or four elements only, at other times very long, consisting of many cells. Individual cells are about 1.0 μ in diameter. This coccus is nonmotile, does not produce spores, is easily stained, and is gram-positive. It grows well upon most laboratory media when sugar (glucose) is present. Growth is much stimulated by the presence of blood serum or of certain growth stimulants. In synthetic media numerous amino acids, glutamine, pantothenic acid, and riboflavine, and perhaps others are required. The colonies developing on agar and gelatin plates are usually small, rarely larger than a pinhead. At first they are transparent, later they become opaque. No pigment is formed and gelatin is not liquefied. When grown upon an agar slant, there is a tendency to form separate colonies rather than for the bacteria to grow together in a continuous mass, as generally occurs with the

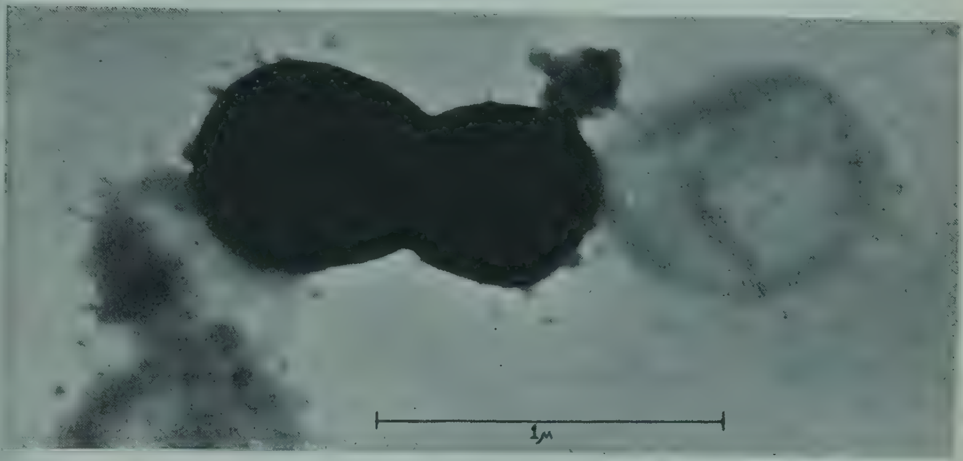

Fig. 31–2. *Streptococcus pyogenes.* Electronograph showing the effect of ultrasonic vibrations. Note that two cells are apparently intact, the cell contents of the others have been dispersed, and the cell walls alone remain, so-called ghost cells. (Courtesy of S. Mudd and D. B. Lackman and the *Journal of Bacteriology.*)

staphylococci. Milk is coagulated by most strains with the production of lactic acid. This last characteristic relates this organism to the *S. lactis* already described among the lactic acid bacteria. Many sugars are fermented with production of acid but no gas. Particularly useful in differentiating this organism from the members of the related genus *Diplococcus* is the lack of ability of *S. pyogenes* to ferment inulin. Nor are the cells soluble in ox bile. *S. pyogenes* is aerobic and facultative anaerobic. It grows best at blood heat, but will also develop at room temperature and below. Sixty degrees Centigrade for fifteen minutes is usually sufficient to destroy this organism, but sometimes strains are encountered which will resist higher temperatures.

Several toxic substances are found in the broth in which *S. pyogenes* has been grown; the amounts and even the kinds may vary with the strain of the organism cultured. Two kinds of hemolysins (called *streptolysins*) have been described, a *leucocidin* which attacks leucocytes, hyaluronidase or spreading factor, and an *erythrogenic* toxin. This toxin differs from most other true toxins in being relatively resistant to heat, even to boiling temperatures. When this toxin is injected intradermally in man, it causes usually the production of a localized redness. Strangely enough, this reaction is apparently characteristic of man and not of the laboratory animals. This toxin, called also scarlatinal or Dick toxin, causes the development of the rash and certain of the disease symptoms characteristic of scarlet fever. Antitoxin can be prepared, but only in relatively low concentration.

Disease Production. There is probably no species of organism which shows more marked variation in virulence than does *S. pyogenes*. In some cases the cultures isolated may show absolutely no power of disease production when injected into suitable laboratory animals such as the rabbit. In other cases, cultures may be so virulent that the introduction of a very few organisms is sufficient to produce fatal results. On the whole, the infections produced by this organism resemble those of the staphylococci, but are usually more severe. It is found that the virulence can be increased by repeated inoculations of animals, and by reisolation from their bodies. Decrease in virulence occurs when the organism is grown for a considerable time upon artificial media. The exact mechanism of disease production is not thoroughly understood.

Streptococcus pyogenes is the cause of many primary infections in man and is also important as a secondary invader. It is sometimes found in wounds, but is not as commonly present as are the staphylococci. Occasionally an unusually virulent streptococcus may gain entrance to the blood stream and cause septicemia or blood poisoning. When growing in a tissue it is possible that it may penetrate a blood vessel wall with the resultant formation of an infected blood clot which may later break up, and when fragments are carried to other parts of the body, the secondary lesions of *pyemia* may be developed. *Erysipelas* is a severe inflammation of the skin caused by the presence of large numbers of these organisms in the lymph spaces of the subcutaneous tissue. It seems to be due to a particular lack of resistance on the part of the individual and unusual virulence on the part of the microorganism. Inflammation of the tonsils (tonsillitis), of the intestines in children (enteritis), or other inflammations of the mucous membranes may be caused by *S. pyogenes*. Puerperal fever, which is primarily an infection of the mucous surfaces after childbirth, commonly followed by an infection of the adjacent tissues and even of the blood stream, is usually due to *S. pyogenes*. It has been found that the organism producing erysipelas is particularly virulent in this respect. Inflammation of the lining membranes of the heart (*endocarditis*) may be caused by organisms gaining entrance to the blood stream and localizing upon the heart valves and adjacent membranes (usually *Streptococcus mitis* is involved). These organisms may produce eventually more or less distortion of the valve, leading to valvular insufficiency. Sometimes cauliflower-like growths occur upon the surface of these valves as a result of irritation by the organisms, and these may break off and be carried by the blood stream to various parts of the body, sometimes effectually stopping the flow of blood to some particular tissues (*embolism*). Invasion of the blood stream may also lead to localization of the organisms in the joints or in the bones, producing one of the many types of disease termed rheumatism (*arthritis*). In a large proportion of cases of endocarditis and arthritis the organisms have gained entrance to the blood stream primarily through the tonsils. Inflammation of the udder (*mastitis*) of the cow is usually produced by this organism or by very closely related forms (as *S. agalactiae*). Milk from such an udder, of course, is not fit for human consumption.

Scarlet fever in man is the result of growth in the mucous membranes of the throat of types of streptococcus capable of producing the erythrogenic toxin already noted. This infection is characterized by the development of a rash on the skin. The fact that streptococci are usually found in the throats of those suffering from scarlet fever has long been known, but proof of their etiologic significance was not clear until demonstrated by G. F. and Gladys Dick. They found that filtrates of broth cultures of this organism when injected intracutaneously give a decided local reaction in a considerable proportion of individuals, but not in those who have been immunized by an attack of the disease. This so-called *Dick* test is used extensively as a means of recognizing non-immune individuals. The characteristic ability of certain strains to produce toxin led investigators to place them in a species, *Streptococcus scarlatinae*. Later it was found that strains of streptococci having different physiologic characters were able to produce the toxin. It is therefore generally assumed that these strains should all be included in the species *S. pyogenes*.

Immunity. Bacterins may be prepared for the treatment and prevention of infection by these organisms in the same manner as has already been described for the staphylococci. Antistreptococcic sera have been prepared by repeated injections of broth cultures of the organism into the horse. Those organisms first injected are previously killed by heat. Later small injections of living organisms are made, and the injections are increased in size until a serum may eventually be prepared that when injected into the body will confer a considerable degree of immunity. However, antistreptococcic serum prepared for one strain of *S. pyogenes* is not commonly efficient in immunizing against another strain. This serum does not owe its curative effect to the presence of antitoxins. Antitoxin for the toxin causing certain of the symptoms characteristic of scarlet fever may be prepared and is effective. The injection of the antitoxin tends to clear up the rash more promptly and to reduce the frequency of complications.

The development of the sulfa drugs and particularly antibiotics has greatly increased the effectiveness of treatment of the streptococcal infections.

Transmission. Inasmuch as this organism is so common upon the surface of the body and in the alimentary tract, it is quite impossible to free the body from it. The strains that are ordinarily found under these conditions, however, are not the most virulent. Sometimes it is necessary that precautions be used to prevent the transmission of virulent strains from one individual to another. The intimate relationship, for example, existing between erysipelas and puerperal fever has already been noted.

Subgroup of Specific Cocci

A number of specific diseases of man and animals are produced by cocci. The most important of these organisms are *Diplococcus pneumoniae,* a cause of pneumonia, *Neisseria meningitidis,* the cause of epidemic cerebrospinal meningitis, and *Neisseria gonorrhoeae,* producing gonorrhea.

These organisms are grouped together inasmuch as they are all cocci and all produce specific diseases. They differ, however, in many other respects, for some are gram-negative (members of genus *Neisseria*), others gram-positive (members of genus *Diplococcus*), some grow much more readily on artificial media than do others.

Diplococcus pneumoniae

Synonyms. *Pneumococcus, Diplococcus lanceolatus, Streptococcus pneumoniae, Micrococcus pneumoniae, Micrococcus lanceolatus.*

This microorganism is a common cause of infectious pneumonia. It was first definitely associated with this disease in 1885 by Frankel and by Weichselbaum.

Morphology and Culture. The organisms usually occur in pairs, rather more rarely in chains of four to six or more individuals. The cells are spherical when isolated, but when in pairs they are generally flattened at the point of contact, with the opposite sides somewhat elongated and pointed. The organism may be readily recognized in stained mounts from the sputum, where it is found to be encapsulated. It stains readily with the common aniline dyes and is gram-positive. Under some conditions in certain culture media it is sometimes difficult to differentiate this organism from the *Streptococcus pyogenes.*

Pneumococci undergo spontaneous autolysis readily. Solubility in bile is a character commonly employed for differentiation of streptococcus and pneumococcus, the latter is readily soluble, the former is not. Sodium taurocholate (10 per cent solution) and certain detergents as sodium lauryl sulfate also cause lysis of pneumococci. In most cases pneumococci ferment inulin with the production of acid, while streptococci do not.

The growth of this organism is never luxuriant upon media, though it will develop on most of them (except potato). The best growth is secured upon rabbit's blood agar. The colonies are α hemolytic, being surrounded by a zone of green. Minute dewdrop-like colonies form upon the surface of the medium, usually not coalescing. Lactic acid is formed from several sugars. Acid is produced in milk, and this medium is coagulated. Growth is better in media to which glycerol and blood serum have been added. The organism grows best at blood heat and will develop little or not at all at room temperatures. It is usually destroyed by direct exposure to sunlight and is killed by drying, although when dried in sputum it may be somewhat resistant.

Serotypes of Pneumococci. The work of Dochez and Gillespie differentiated several distinct types of the pneumococcus, the differentiation being based primarily on the immunological reactions, particularly agglutination. These were termed types (better, *serotypes*) I, II, III, and IV. The first three were relatively distinct, the fourth being constituted to contain those organisms not belonging to one of the other types. Later the situation was found to be even more complex. Cooper and associates have broken up the original type IV into 29 additional serotypes. Other workers have added to the list of serotypes, and to make the situation more complex, subdivisions or subtypes were made within serotypes, until the list has swollen to 75 or more. This ability of *Diplococcus pneumoniae* to show itself in numerous forms would be of academic interest only were it not for the fact that the determination of the serotype in a particular case of the disease is prerequisite to proper therapeutic use of antiserum. A brief survey is therefore needed.

Pneumococci produce two kinds of antigens. All pneumococci produce the same *somatic* antigen, apparently a constituent of the cell protoplasm. But the cells of pneumococci are surrounded by a mucilaginous or capsular layer which may be of any one of many kinds. These capsular substances are all polysaccharides, related to plant gums. They are termed specific soluble substances (abbreviated as SSS). The SSS reacts with specific antibodies so that the different kinds may be differentiated by use of homologous agglutinins or precipitins. Serotype differentiation is therefore largely a matter of identifying the large number of specific soluble substances.

Pneumococci as usually studied are in the smooth (S) stage. It is possible to secure the rough (R) stage or variants. These latter produce a rough colony instead of a moist, mucoid colony, and microscopically are found to be free of capsular material; the cells have lost the power of producing the characteristic SSS. They no longer show serotype specificity.

Three methods of determining the serotype of a culture of pneumococcus have come into use. Antisera are prepared by injection of suitable animals (rabbit) with the several serotypes. Each animal should yield a serum containing antibodies specific for the SSS of the serotype injected. These antisera are used for the identification of the serotype of cultures of pneumococci isolated from clinical cases of disease, such as from the sputum of pneumonic patients. The culture to be diagnosed is tested against each of the various antisera, in one or more of three ways: (1) the ability of the antiserum to agglutinate a suspension of the organism may be used; (2) or the antiserum may be added to the washings from the bacteria with resultant precipitation of the SSS; (3) or a mixture of the bacteria and undiluted antiserum may be watched under the microscope for the appearance of the *swelling reaction* (frequently referred to by its German name, *Quellungs reaction*). A positive reaction is manifested by a marked swelling of the capsule without any change in the size of the enclosed organism. In order to cut down the number of separate tests to be made one may mix (pool) several sera, as many as six, and test the organism to determine which pool contains the specific antiserum, and then test with each of the individual antisera represented in the pool.

Disease Production. The pneumococcus seems to be present normally in the mouths of a considerable percentage of individuals. If sputum from such an individual is injected into a guinea pig, or better, intravenously into a rabbit, the animal will frequently die of an acute septicemia, and the organism can be demonstrated without difficulty in the blood.

Pneumococci are the most common cause of lobar pneumonia. The tissues of the portion of the lung invaded by the pneumococcus become congested with blood, and blood plasma passes out into the air sacs (*alveoli*). Here the fibrinogen coagulates (is converted into fibrin), and that portion of the lung becomes *hepatized,* or liverlike, in consistency. Sometimes red blood corpuscles likewise find their way into these alveoli, and a section through the lung tissue shows it to be red in consequence. The leucocytes later invade the tissues and air sacs, and they become gray. The microorganisms multiply in the tissues and alveoli, and in some cases gain entrance to the blood stream, producing a septicemia. The damage done to the lung tissue itself does not seem to be sufficient to account for the grave disturbances in general health characteristic of this disease. It is possible that poisons, probably endotoxins, are produced in sufficient quantities by this organism to account for the fever. During convalescence the fibrin and other material in the air sacs undergo autolysis as a result of digestion by enzymes secreted by leucocytes, possibly also by the lung cells.

The pneumococcus may sometimes invade parts of the body other than the lungs and blood stream, producing such conditions as pleurisy, empyema, meningitis, endocarditis, and arthritis.

Immunity. It is probable that recovery from the disease is largely due to the production of opsonins in the blood and tissues as a result of the presence of the organism. Immunity against pneumonia is short. As a matter of fact, complete recovery is sometimes followed by an increased susceptibility to the disease. Antipneumonic serum has been prepared by the systematic injection of killed and living cultures of the pneumococcus into animals. Its use is complicated by the fact that antiserum prepared by use of one type of pneumococcus is not effective against infections produced by other types. In fact, the antiserum specific for type I pneumococcus is the only one which has proved particularly effective in man.

The pneumococcus has a complicated series of immunological relationships. The capsular material of each type is composed of a different polysaccharide material which upon hydrolysis yields sugars and uronic acids. The possession of the capsule apparently interferes with the functioning of the normal mechanism of the body defense. Efforts have been made to secure enzymes from other microorganisms which would dissolve or digest these specific capsular materials. Bacteria have been found (in soil) which produce such enzymes, but they have not proved to be therapeutically significant.

Recent advances in chemotherapy and development of antibiotics have aided materially in combating pneumonia.

Transmission. Pneumonia is not usually regarded as a contagious disease, although it is undoubtedly true that when a number of individuals having a low grade of resistance are associated together, the disease sometimes assumes epidemic form. It is probable that in most cases the organisms responsible for the disease are already present in the mouth and throat and begin to develop as a result of a diminution of natural body resistance.

Neisseria meningitidis

Synonyms. *Diplococcus intracellularis meningitidis, Micrococcus weichselbaumii; Streptococcus meningitidis*, meningococcus.

The organism is the specific cause of the disease known as epidemic cerebrospinal meningitis in man, sometimes also called spotted fever.

The meninges are the membranes which cover the brain and spinal cord; cerebrospinal meningitis, therefore, is the name applied to an acute inflammation of these membranes. The organism was first cultured and described by Weichselbaum in 1887.

Morphology and Culture. Material for an examination of the organism as it occurs in the exudate from the meninges may be obtained by inserting a hypodermic needle between two of the lumbar vertebrae. The fluid is under sufficient pressure so that a few drops may be collected upon glass slides or used to make cultures. In stained preparations of the fluid the organisms are found within the white blood corpuscles or phagocytes. They usually occur as diplococci or in groups of fours. When grown on culture media, the cells are about 1 μ in diameter, usually in pairs, sometimes in chains. The organism is easily stained and is gram-negative. This fact differentiates it sharply from the pneumococcus previously described.

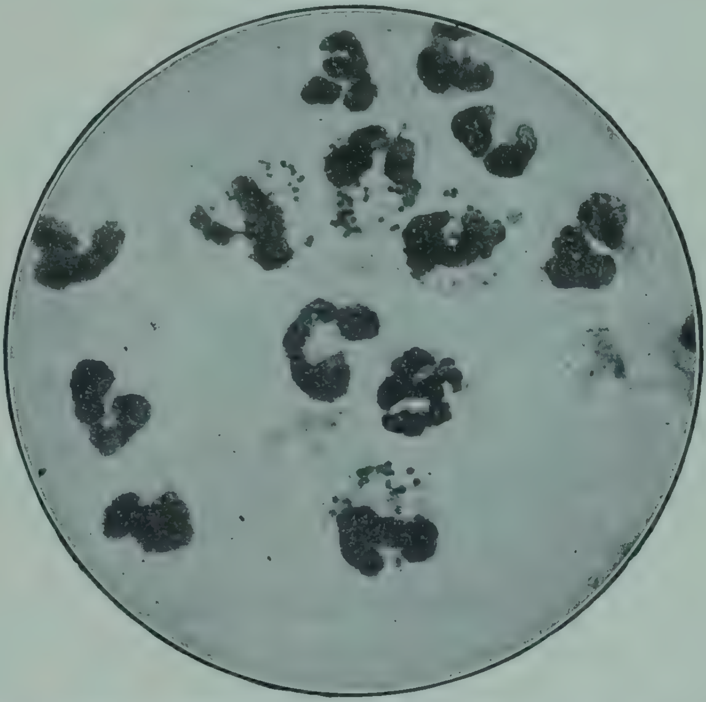

Fig. 31–3. *Neisseria meningitidis,* in a preparation of pus from a brain abscess (Flexner).

The meningococcus, when first isolated, does not grow readily upon culture media except in the presence of blood serum. Upon this medium a white viscid coherent colony is developed. On blood agar there is no evidence of hemolysis. The meningococcus does not liquefy gelatin, nor change milk. It produces acid from dextrose and maltose, but not from sucrose or levulose. Ability to ferment maltose is one character differentiating this organism from the gonococcus. Cultures passed through several transfers tend to become more luxuriant on media. It does not retain its vitality for long periods upon artificial media as the cultures quickly autolyze. Frequent transfers are therefore necessary to maintain the cultures in vigorous condition. It is destroyed very quickly by desiccation and by the action of disinfectants and sunlight.

Classification of Meningococci. As with the pneumococcus, it has been found possible and advantageous to classify meningococcus cultures into several serotypes. Originally Gordon and Murray distinguished four serotypes. Those commonly encountered are now known as group I and types II and IIa.

The antigens of the meningococcus for which antibodies, such as precipitin, may be prepared are (1) polysaccharides characteristic of the several types, (2) a second polysaccharide found in all meningococci and in the gonococcus as well, and (3) a protein substance common to all meningococci.

Disease Production. This organism does not readily produce disease in laboratory animals unless injected in large quantities. However, inoculation of some animals, as the monkey, causes the development of disease symptoms practically the same as in man. The organism grows upon the surface of the meninges, causing an acute inflammation with a purulent exudate. The manner in which the organism gets into the brain and spinal cavities in man is not entirely clear. It has been found on the nasal mucous membranes of those having the disease and of those associated with such individuals. It is probable that the organism gains entrance in some manner to the blood stream and finds favorable conditions for its development on the meninges; possibly it enters through lymph channels. Unless appropriately treated, the disease is highly fatal. Until the introduction of the serum noted below and the more recent sulfa drugs and antibiotics, few cases recovered.

Immunity. An immune serum has been obtained by the repeated injection of the horse with pure cultures of this organism. The blood serum is used. Several cubic centimeters of the fluid surrounding the meninges of the patient are removed by means of the hypodermic needle as explained above. About the same amount of the immune serum is injected to take its place. The fact that the immune serum comes into immediate contact with the organisms on the meninges seems to give optimum conditions for its action, and very favorable results have been reported as a consequence of its use.

The problem of immunization against meningitis is complicated (as in pneumonia) by the existence of several distinct types of meningococci. The difficulties may to some degree be obviated by the use of a polyvalent serum, that is, one which has been prepared by immunizing a horse (usually) against several types simultaneously.

Transmission. It is probable that the disease is transmitted from one individual to another by more or less intimate contact, the use of common drinking cups, mouth spray, etc. The disease sometimes appears in epidemic form and is usually confined to children, although adults are not entirely immune.

The disease is rather difficult to combat because of carriers. The organism apparently first invades the naso-pharynx, where it finds conditions suitable for growth. In a considerable percentage of these the organisms never invade the meninges, but remain localized at the initial site of infection. Such individuals are carriers, and their recognition is a matter of considerable importance in controlling an epidemic of the disease. Carriers are recognized by isolation of the characteristic organisms from the throat.

Neisseria gonorrhoeae

Synonyms. *Diplococcus gonorrhoeae, Micrococcus gonorrhoeae,* gonococcus.

The gonococcus is the specific cause of gonorrhea and its related disorders in man. Gonorrhea is primarily a disease of the mucous membranes of the urogenital tract. In some cases the membranes of the eye may be infected. It is one of the most widespread and serious of human diseases. The organism was first observed in gonorrheal pus by Neisser in 1879, but it was not cultivated until 1885 when methods were developed by Bumm.

Morphology and Culture. The gonococci in stained mounts of gonorrheal pus are usually to be found within the white blood cells. Typically they occur in pairs, flattened on the proximal ends and rounded at the distal ends. They may be described as coffee bean-shaped. The cells when free are spherical. They are about 1.6 μ by 0.8 μ. Chains are rarely formed in culture media. The cells stain readily with the aniline dyes and are gram-negative. This point is of considerable importance in the diagnosis of infections, as most other pus-producing cocci are gram-positive. The organism is cultivated with difficulty when first isolated in pure cultures, even upon a serum medium. After cultivation on serum agar and a succession of transfers, growth becomes more luxuriant. The growth consists of minute, discrete, transparent colonies. The gonococcus may be differentiated from the morphologically similar meningococcus by the fact that it produces acid from dextrose only.

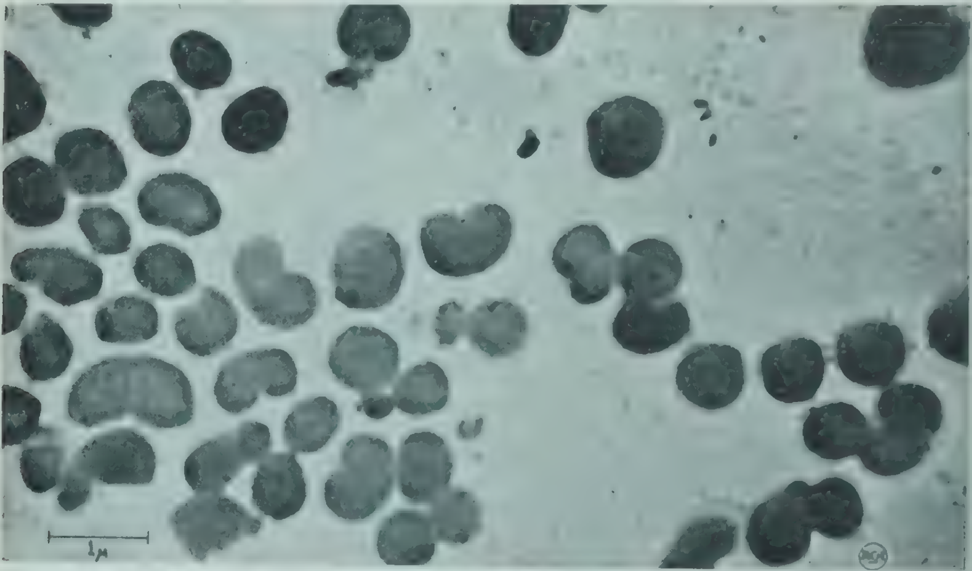

Fig. 31–4. *Neisseria gonorrhoeae.* Electronograph. Shows many irregular forms. (Courtesy of G. Knaysi and S. Mudd and the *Journal of Bacteriology.*)

The gonococcus is aerobic, easily destroyed by drying, and even in culture media frequent transfers are necessary to keep it alive. The older cultures quickly undergo autolysis. It grows only at temperatures within a few degrees of blood heat. It is readily destroyed by disinfectants and by heat. The possibility of transference of the infection other than by direct contact is rather remote, owing to the readiness with which the organism succumbs to unfavorable environment.

Disease Production. Gonorrhea is a disease characteristic of man alone. It has not generally proved possible to transmit the typical disease to animals, although local suppuration and necrosis and even death may be occasioned by injections of the organisms. Man has been infected by the use of pure cultures, so that there is no question as to the causal relationship of the organism to the disease.

In the male the primary infection is manifested by an inflammation of the urethra. Quite characteristic is the production of a thick, creamy pus. The inflammation may extend to the prostate gland, the bladder, the seminal vesicles, and even to the epididymis. After a time the secretion changes in character, becomes serous, and may cease. The disease may persist in chronic form. Long-continued inflammation may lead to the formation of scar tissue, and constriction of the urethra, or stricture. The organism sometimes spreads to other parts of the body, particularly the joints, with consequent production of gonorrheal rheumatism; more rarely it may cause endocarditis. The disease may persist in latent form for a considerable period of time, even years, after the initial infection, and may recur in acute form.

In the female the initial infection is usually of the vulva. It may spread to the urethra and bladder, more commonly to the uterus. A not infrequent sequel is involvement of the Fallopian tubes, requiring surgical intervention and consequent sterilization. Blindness resulting from inflammation of the eyes and adjacent membranes of the newborn is usually the result of gonorrheal infection of the mother. When there is reason to suspect such infection, it is customary to instill antiseptic solutions, usually silver compounds, into the conjunctiva of the child to prevent the development of the organism.

Particularly to be emphasized is the long period of time during which an infected individual may be able to transmit the infection, even though there has apparently been complete recovery.

Immunity. Very little if any immunity is conferred upon an individual by an attack of gonorrhea. Satisfactory methods of passive immunization have not been developed, perhaps in part due to the multiplicity of strains or types. Vaccines consisting of suspensions of gonococci killed by heat have proved useful, particularly in chronic infections. The disease, after it has passed the acute stage, is difficult to cure by the local use of disinfectants. Antibiotics are used successfully.

Transmission. The disease is transmitted in the great majority of cases by contact. Epidemics have been observed, however, in children's hospitals due to the common use of towels, washbasins, etc. There is a possibility of such transmission in public lavatories and toilets, although infection in this manner is undoubtedly rare.

CHAPTER 32

Diphtheria Group: The Genus *Corynebacterium*

The organisms belonging to this group are gram-positive bacilli which when properly stained usually show characteristic metachromatic granules. They are non-motile and do not produce spores. The most important organism belonging to the group is *Corynebacterium diphtheriae*. Some organisms related to *C. diphtheriae* have been described under the name *C. pseudodiphtheriticum*. They differ in some degree morphologically from the true diphtheria bacillus and are not capable of producing disease. The term *diphtheria* as originally used by physicians indicated a pathologic condition in which there was more or less extensive death of the cells of the mucous surfaces and formation of false membranes of fibrin, usually in the throat or nose. This type of inflammation was said to be *diphtheritic*. It has been found to be most commonly caused by *C. diphtheriae*, but other organisms have been described which can bring about somewhat similar changes. The term *diphtheria* as commonly used at present indicates a diseased condition in which the specific organism, *C. diphtheriae*, is present, even though the typical false membrane is not produced.

Corynebacterium diphtheriae

Synonyms. *Bacillus diphtheriae, Bacterium diphtheriae, Mycobacterium diphtheriae.*

This organism is the cause of the disease diphtheria in man. The disease is not to be confused with somewhat similar diseases in animals sometimes termed diphtheria; these are caused by organisms which are quite distinct. *Corynebacterium diphtheriae* was first described by Klebs in 1883, and Loeffler in 1884 studied it in pure culture. Roux and Yersin between 1888 and 1890 demonstrated that it produces a characteristic toxin and that many of the symptoms and lesions of diphtheria can be duplicated in laboratory animals by the injection of bouillon filtrates.

Morphology and Culture. When grown on culture media, the diphtheria bacillus shows such great variations in its morphology that some writers believe it to be more closely related to fungi than to the true bacteria. For example, club-shaped and branching forms are not uncommon. Typical organisms when stained with methylene blue are seen to be rods varying from 0.4 to 1.4 μ in diameter and from 1.5 to 5.0 μ or even more in length. Sometimes they stain uniformly, but more commonly show deeply stained bands or granules (termed metachromatic granules) in the cell which give to the cell contents a mottled or barred appearance. The organism does not produce spores or capsules, is non-motile and gram-positive. Cultures may be secured upon blood serum slants directly from the throat of a patient with diphtheria. The diphtheria organism kept at blood heat upon this medium grows at first more rapidly than most of the other bacteria that may be present. It forms upon the surface of this medium, sometimes within twelve to eighteen hours, small pinpoint, translucent dots which later became opaque, gray colonies. It does not grow very readily, at first at least, upon agar and gelatin unless glycerol is added to them. After cultivation for some time on artificial media, development upon

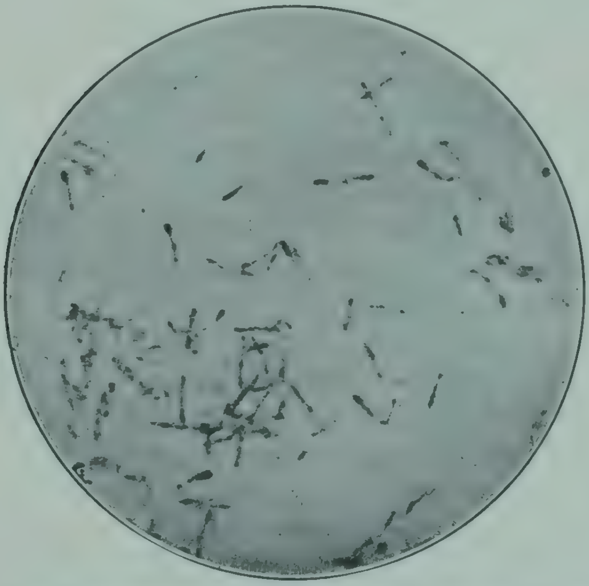

Fig. 32–1. *Corynebacterium diphtheriae.* (After Epstein in *Journal of Infectious Diseases.*)

agar or gelatin is somewhat more luxuriant. It produces no observable change in milk. In bouillon after a time a delicate film will form, and transfer of this film to new tubes of broth will confine the growth practically to film formation. This, it will be remembered, is the method employed in the manufacture of diphtheria toxin.

Special media allowing the growth of the diphtheria bacilli but inhibiting most other organisms are often used in diagnosis of the disease.

Corynebacterium diphtheriae is aerobic. It retains its vitality for a long time when grown upon culture media. It is not particularly resistant to drying, but when in sputum or diphtheritic membrane it may be dried for considerable periods, in some cases, months, without being killed. The temperature of pasteurization of milk is sufficient to destroy the organism. Gelatin is not liquefied.

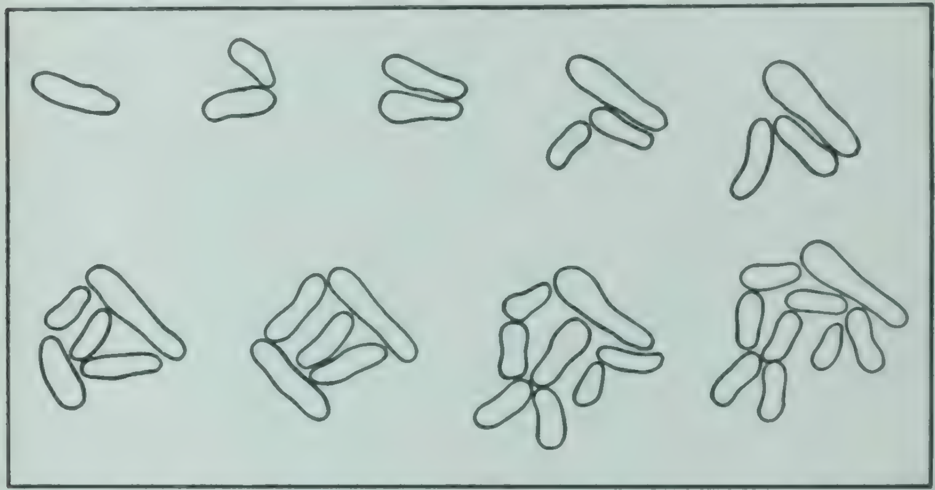

Fig. 32–2. The "snapping" method of cell division in diphtheroid bacilli. There is some tendency for the daughter cells to lie parallel to the mother cell. Note that the top cell undergoes no division. (Adapted from Graham-Smith.)

Disease Production. Diphtheria is typically a disease of man, particularly of children. It is a true toxemia, that is, the organisms are confined to a relatively small area of the mucous membrane of the nose or throat, and growing there, produce their characteristic toxin, which is absorbed by the blood and injures various internal organs, such as the liver and the heart muscles. The false membrane as it occurs in the throat results from the direct injury to the mucous membranes by the organisms and their toxin, causing death of the cells, and a considerable degree of inflammation and swelling consequent upon the inflammation of the tissues. The blood vessels are engorged with blood, and blood plasma is thrown off. The fibrinogen is converted rapidly into fibrin and, when mixed with desquamated cells of the mucous membrane and red and white blood corpuscles, constitutes the false membrane. In this membrane conditions are usually quite favorable for the continued growth of the organism. It sometimes happens that the membrane becomes large enough to cause death by asphyxiation due to the occlusion of the air passages. The pharynx is most commonly affected; the larynx and the nasal mucous membranes may also be involved. Occasionally the organism may invade the middle ear through the Eustachian tube. Sometimes the diphtheria bacillus may develop in wounds.

Diphtheria Toxin. The production of diphtheria toxin was considered briefly in the discussion of some of the general problems in immunity. Diphtheria toxin is one of the most powerful poisons known. When produced under most favorable conditions the broth medium may contain as many as a thousand minimum lethal doses of toxin per milliliter, that is, a thousand times as much as necessary to kill a guinea pig. The toxin may be produced in a medium in which all the constituents are known chemically and is found to be much more complex than any of the medium constituents. It gives the reactions of a protein with a molecular weight of about 72,000. It is produced only under aerobic conditions and in a medium which is slightly alkaline in reaction. The different strains of bacteria that have been isolated may show great variations in ability to produce toxin, varying from those producing none at all to those which manufacture it in large amounts. The strain that has been used widely in the commercial preparation of the toxin is one known as the Park strain, named for Dr. Park who first isolated it. It has been found that it is necessary to have a concentration of iron in the medium within relatively narrow limits if toxin production is to reach its maximum. The toxin is apparently the protein portion of a respiratory enzyme which contains iron. When there is an adequate supply of iron in the medium, the complete non-toxic enzyme is synthesized.

Immunity. A person who has recovered from diphtheria is thereafter relatively immune to the disease, at least for a time. This is undoubtedly due in part to the development in the body of antitoxins which neutralize the poison. In treatment of the disease diphtheria, antitoxic serum prepared from the blood of immunized horses is injected. It may also be used for the passive immunization of individuals exposed to the disease.

Active immunization to diphtheria is extensively practiced, particularly in children. Two properly spaced injections are made of a toxin which has been so treated as to lose its poisonous characteristics, but not its antigenic properties, to convert it into toxoid. Usually a reasonably solid or lasting active immunity is conferred by this procedure. Several methods of transforming toxin to toxoid have been used, perhaps the most common being prolonged treatment with formaldehyde.

A mixture of toxin and antitoxin, such that the toxin is very nearly neutralized, has also been used with success.

Diagnosis. Most states and cities maintain bacteriological laboratories to assist physicians in diagnosing such diseases as diphtheria. The method consists in passing a sterile swab over the surface of the inflamed area and drawing this over the surface of some suitable culture medium, usually Loeffler's blood serum. The culture so obtained is then sent or taken to a laboratory and incubated from twelve to eighteen hours. Mounts stained with Loeffler's methylene blue show the characteristic organisms with metachromatic granules. It is sometimes possible to make diagnoses of diphtheria from stained mounts of material taken directly from the throat.

The *Schick test* is frequently used in an effort to determine susceptibility to diphtheria. The test consists of the injection of a minute dose (one-fifteenth of the dose lethal to a guinea pig weighing half a pound) of diphtheria toxin into the skin. If the blood of the individual tested contains more than a minimal amount of antitoxin, there is no reaction. Susceptibility to diphtheria is shown by reddening and swelling of the tissues.

Transmission. The most common method of transmission of diphtheria is by the use of common drinking vessels and by putting contaminated articles such as lead pencils in the mouth. It may sometimes be contracted by the inhalation of infective droplets. Several epidemics have been shown to be due to milk transmission. Contamination of the milk arises from the fact that the organism gains entrance to the milk from some individual who handles it, and not directly from the cow. Carriers who harbor the organism but show no clinical symptoms of the disease are one of the major problems in the control of the disease.

Other Species of Corynebacterium. There have been many proposals to divide *C. diphtheriae* into two or more species. Bacteria closely resembling the true diphtheria organism, but non-pathogenic and usually not producing acid from glucose and sucrose, have been placed in the species *Corynebacterium pseudodiphtheriticum*. The true diphtheria cultures were placed by McCleod in three groups which seemed to correlate colony characters on a special blood medium containing potassium tellurite. Those strains which were predominantly pathogenic, on the special medium produced dark gray colonies with raised center and radially striate margins (so-called daisy head colonies) fermenting glycogen and starch, and which were non-hemolytic were designated as *C. diphtheriae* var. *grave*. The strains of the second group, which produce convex, black, entire colonies, do not ferment starch and glycogen and are hemolytic, were given the varietal designation *mite*. The strains of the third group, which produce a small, flat colony with a black, raised center, not hemolytic, and which do not ferment starch or glycogen, were termed var. *intermedium*.

Certain animal diseases are produced by distinct species of the genus *Corynebacterium*. Suppurative infections in cattle, sheep, and swine are often caused by *C. pyogenes*. A mouse septicemia is produced by *C. murisepticum*.

CHAPTER 33

Plague Group: The Genus *Pasteurella*

The organisms belonging to the plague or hemorrhagic septicemia group of bacteria are all short, oval rods, non-motile, non-spore-producing, and gram-negative. When grown in the body (sometimes also in culture media) they show *polar granules* if stained by appropriate dyes. To this group of organisms the generic name *Pasteurella* is usually applied. The organisms causing several diseases of animals are included in this group. Among these are the hemorrhagic septicemias of cattle, swine, rabbits, and fowls. Two species are known to produce disease in man, the *Pasteurella pestis*, the cause of plague, and *P. tularensis*, the cause of tularemia. To the causal organisms of hemorrhagic septicemias the name *P. multocida* has been given.

Pasteurella pestis

Synonyms. *Bacillus pestis, Bacillus pestis bubonicae, Bacterium pestis.*

This organism was described independently by Yersin and by Kitasato in 1894 as the cause of bubonic plague. The disease is one which is endemic in parts of China, and possibly in India as well. History shows that at several different times an epidemic of this disease has spread over the entire civilized world. Such, for example, was the "black death" which devastated Europe. In the past several decades the disease has been transported to practically every port of prominence in the world. It has not succeeded generally, however, in maintaining itself in most of these countries, but during the first decade of the present century it gained a foothold in the rats and later in the ground squirrels and other rodents in California and has gradually spread eastward as far as the Rocky Mountains, or perhaps farther.

Occasional cases of plague in man develop as a result of contact with these rodents. Apparently the United States has been added to China as an area in which plague is endemic, for a small number of cases in man appear almost annually.

Morphology and Culture. *Pasteurella pestis* is a small non-motile rod 0.5–0.75 μ by 1.5–2 μ. It is usually single, rarely occurring in chains. In certain culture media involution forms are commonly observed. When smears are made directly from infected tissue, the organism exhibits a very decided bipolar staining. It may be easily isolated from infected lymph glands in pure culture. Occasionally it may be obtained from blood. It grows readily upon various culture media, but poorly, or not at all, upon potato. It has an optimum temperature between 25° and 30° C. It is only slightly resistant to heat, drying, light, or disinfectants.

Disease Production. Bubonic plague has come to be regarded as a disease characteristic of certain rodents, particularly the rat and certain related forms. It is readily transmitted to man. Mice, rats, guinea pigs, rabbits, and other rodents may be readily infected. The disease as it occurs in rodents in nature is termed *sylvatic plague*.

The disease in man manifests itself usually in one of three types, depending apparently upon the method of infection and virulence of the organism. The organism may enter through the skin and cause the production of buboes, that is, swellings of lymph glands, which frequently ulcerate and discharge pus superficially. These are the characteristic lesions of bubonic plague. This stage is frequently followed by invasion of the blood stream and consequent septicemia. In some cases the organism is sufficiently virulent to invade the blood stream almost immediately, with the production of the septicemic type of plague. Usually in this disease there are hemorrhages in the subcutaneous tissues, with consequent formation of darkened areas of skin. This is characteristic of the hemorrhagic septicemias. It was this darkening of the skin which gave the name "black death" to the disease. The third type of the disease, the pneumonic, is probably usually contracted by the inhalation of infectious droplets. It is an acute and quickly fatal type of pneumonia.

Immunity. Those who recover from an attack of plague show a relatively high degree of immunity. The organism does not produce a toxin. Antisera have been prepared, but the results obtained have not always been favorable. The antiserum is prepared by repeated injections of killed cultures into a suitable animal, such as the horse, followed by increasing doses of living bacteria. Blood serum from such an animal which has developed a high degree of immunity is believed to confer a considerable degree of passive immunity upon the individual into whom it is injected. Active immunization by the injection of killed or even living attenuated cultures of bacteria has been extensively practiced and has proved successful.

Transmission. The disease may be transmitted by the inhalation of infectious droplets, or by close contact with infected individuals. The more common method of transmission, however, is by the bite of infected fleas or through their excretions scratched into the skin. It has long been known that an outbreak of bubonic plague is frequently preceded by the appearance of the disease among rats and other rodents. It is now known that rat fleas will leave the body of a rat which has died and may attack man. The organism multiplies in the alimentary tract of the fleas. Bubonic plague is evidently a vermin disease.

Pasteurella tularensis

Synonym. *Bacterium tularense.*

A disease somewhat resembling plague was discovered by McCoy and Chapin in 1912 in a California ground squirrel. Later it was found that the disease is rather widespread among rabbits, and perhaps other rodents, almost throughout the United States. It has also been reported from Japan and other countries. It was found to be the cause of so-called deerfly fever in the western part of the United States, apparently transmitted to man by flies that had previously bitten infected jack rabbits. Later numerous cases came to light in which the infection came from handling rabbits which had been shot. The disease has been named tularemia, after the organism, and this in turn after Tulare County in California, where it was first discovered.

Morphology and Culture. *Pasteurella tularensis* is a minute gram-negative rod which in laboratory media exhibits a marked degree of pleomorphism. It is grown with some difficulty in media. Those which have proved most satisfactory are coagulated egg yolk and a blood glucose cystine agar. Acid (no gas) is produced from dextrose, levulose, mannose, and glycerol.

Disease Production. Tularemia usually manifests itself by the development of ulcerated lymph nodes, usually those near the site of inoculation. In this respect it resembles somewhat the bubonic plague. In some cases the disease is somewhat typhoid-like in its manifestations. It is not usually fatal. A relatively permanent immunity is acquired as a result of recovery.

CHAPTER 34

The Intestinal Group or Enterobacteria

THE GENERA Escherichia, Aerobacter, Salmonella, Shigella

The organisms included in the enterobacteria, as the name indicates, are characteristic members of the intestinal flora in health or disease. Some are also found living saprophytically in soil and water. They are all plump, gram-negative rods, and do not produce spores. All are aerobic and most are facultative anaerobic. Some species are motile, others non-motile. This group is of importance because of several disease-producing bacteria that it contains, also because of the fact that the intestinal bacteria as has already been noted are the ones for which search is made in an examination to determine the potability of water.

The naming of the organisms of this group is not satisfactorily stabilized. Some authors have considered all the organisms to belong to a single genus *Bacterium*, others have divided the enterobacteria into a large number of genera.

The group may be divided into three subgroups, the basis for the classification being the ability of the organisms to ferment various sugars. The organisms of subgroup I ferment both dextrose and lactose with formation of both acid and gas, and constitute the *coliform* subgroup; those of subgroup II produce both acid and gas from dextrose but neither from lactose and are termed the *intermediate* subgroup; and those of subgroup III may or may not form acid from dextrose but usually not from lactose, and gas is produced from neither of the sugars, termed the *typhoid-dysentery* subgroup.

These subgroups have been modified in recent years by the emphasis which has been laid upon classification by use of serologic tests. The principal result has been the inclusion of the typhoid organism in the intermediate subgroup in the genus *Salmonella* in spite of its lack of ability to produce gas from dextrose, and the Sonne bacillus in the genus *Shigella* even though it produces acid from lactose.

SUBGROUP I. THE COLIFORM OR COLON-AEROGENES SUBGROUP

This subgroup includes several species, for the most part non-pathogenic, many of them inhabitants of the intestines of man and animals, and some which may develop saprophytically. Those which are parasitic primarily are of great interest inasmuch as their presence or absence in water is one of the best evidences of sewage contamination. Those which are non-parasitic are of significance because they may be confused with the preceding. All produce both acid and gas in the fermentation of both dextrose and lactose.

The organisms of this group are usually divided into at least two genera, *Escherichia* and *Aerobacter*. The two genera may be differentiated by reference to the following diagnoses:

Escherichia (Bacterium). Gas produced from dextrose has a carbon dioxide-hydrogen ratio of 1–1. Acetoin (acetyl methyl carbinol) is not produced from dextrose, hence the Voges-Proskauer test is negative. When grown in a special dextrose medium the hydrogen ion concentration is increased to a point such that the methyl red indicator shows its acid color red, i.e., the organisms are said to be methyl red positive. In general the bacteria of this genus do not utilize citric acid as a source of carbon. It is probable that by international agreement the generic name *Escherichia* will be replaced by the older name *Bacterium*.

Aerobacter. Gas produced from dextrose has a carbon dioxide-hydrogen ratio of 2–1. Acetoin is produced, i.e., the organisms are Voges-Proskauer positive. They are methyl red negative and utilize citric acid.

Occasionally organisms are encountered which are intermediate in one or more characters, but usually cultures of organisms of this subgroup can be allocated definitely to one genus or the other. The genus *Citrobacter* is sometimes recognized to include certain species which show characteristics intermediate between *Escherichia* and *Aerobacter*.

Each genus contains several species which may be differentiated on the basis of fermentative and other reactions. The type of each genus (*Escherichia coli* and *Aerobacter aerogenes*) only will be discussed.

Escherichia coli (Bacterium coli)

Synonyms. *Bacterium coli, Bacillus coli communis, Bacterium coli commune,* colon bacillus.

Escherich in 1886 isolated the organism from normal feces and called it *Bacterium coli commune*. The organism is quite commonly present in the intestinal tract in man and most animals. Whether or not it ever occurs in nature wholly independent of fecal contamination is an unsettled question. Undoubtedly it may continue to grow for some time outside of the body under favorable conditions, but it does not seem to be common, at least not in virgin soil and unpolluted water. Some of the older reports of finding this organism in nature under conditions which made improbable fecal contamination were based upon confusion of this organism with *Aerobacter aerogenes.* Its presence in water is universally regarded as indicative of sewage pollution. Such water is not regarded as potable. This is not because *Escherichia coli* possesses of itself any marked pathogenic characters, for it is found in greater numbers in the normal human intestines than in grossly polluted water, but because its presence indicates sewage pollution and the possible, even probable, presence of disease-producing bacteria such as the typhoid bacillus.

Morphology and Culture. *Escherichia coli* is a plump rod 0.4–0.7 μ by 2.0–4.0 μ. Occasionally it is shorter, and when growing rapidly may resemble a coccus. It is usually single, but may form short chains. Neither spores nor capsules are produced. It is motile in young cultures, although never actively so. It stains readily and is gram-negative.

Upon gelatin plates the colonies are moist, grayish white, opaque, becoming darker and coarsely granular. Iridescent green colonies are formed on eosin methylene blue agar. Gelatin is not liquefied. In gelatin stabs a uniform filiform growth occurs along the line of puncture, and the growth spreads over the surface to some extent. Bouillon is clouded, sometimes with the formation of a membrane. A moist, spreading growth appears upon the potato, and the medium is darkened, sometimes becoming almost black. Milk is coagulated with the formation of lactic acid and gas. The curd shrinks, but is not digested, as this organism is not actively proteolytic.

Escherichia coli is an aerobe and in the presence of carbohydrates a facultative anaerobe. It grows best at blood heat, but develops well at room temperatures and even below. It is destroyed when heated to a temperature of 60° C. for 15 minutes. Many carbohydrates are fermented, among them dextrose, lactose, and maltose. Other closely related strains ferment sucrose. Indole is formed in peptone solution. Acetyl methyl carbinol is not formed from dextrose, that is, the Voges-Proskauer test is negative.

This organism is not usually regarded as pathogenic, although many authors have ascribed disease-producing powers to it. This is due in part to the fact that they have not discriminated carefully between *Escherichia coli* and some of the other organisms belonging to the intestinal group. However, when broth cultures of this organism are injected intraperitoneally into a guinea pig, the animal dies, usually within three days. In man it has been known occasionally to invade the gall bladder. It has also been described as producing inflammation of the urinary bladder.

Methods used in the isolation and identification of this organism in water analyses have been discussed in Chapter 25.

Aerobacter aerogenes

Synonyms. *Bacterium aerogenes, Bacterium lactis aerogenes, Bacillus lactis aerogenes.*

This organism was first described by Escherich in 1885 from souring milk. Since that time it has been found repeatedly under the same conditions as *Escherichia coli,* that is, it has proved to be one of the common intestinal organisms. Recent work seems to show that this organism, unlike *Escherichia coli,* is not uncommon in nature outside the alimentary tract. It has been found repeatedly in soils and on grains, likewise it is abundant in water of streams during floods and high water. Its relationship to the souring of milk has already been discussed.

Morphology and Culture. Morphologically *Aerobacter aerogenes* differs from *Escherichia coli* principally in the total absence of flagella, i.e., the organism is non-motile. Under suitable conditions it is able to produce capsules when grown in milk. The colonies upon agar and gelatin are thicker, larger, and more slimy than those of the colon bacillus, and milk is curdled even more rapidly. This organism ferments dextrose, lactose, sucrose, and starch with the production of both acid and gas. Cultures upon potato generally show gas bubbles. Indole is formed in peptone solution. Acetyl methyl carbinol is produced from dextrose, i.e., the Voges-Proskauer test is positive.

Disease Production. This organism is not known to be pathogenic.

In water analyses it is frequently confused with *Escherichia coli*. It does not apparently have the same sanitary significance as *E. coli*.

SUBGROUP II. INTERMEDIATE OR SALMONELLA SUBGROUP

Here are included those organisms which belong to the intestinal group and which ferment dextrose, but not lactose, with production of acid and gas (usually). Two genera may be differentiated, *Proteus* and *Salmonella*.

Proteus. Acid and gas produced from sucrose, gelatin usually liquefied. The most common species, *Proteus vulgaris*, has been reported as occasionally pathogenic. It is not of sufficient importance to be discussed further here. Its relationship to decay has been previously considered. One strain has an interesting relationship to the diagnosis of typhus fever (Chapter 46).

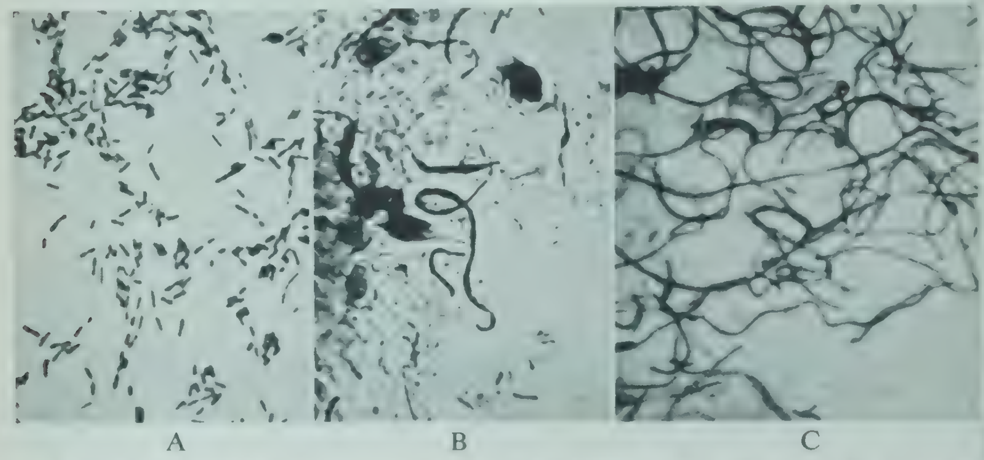

Fig. 34–1. *Proteus*. Photomicrographs. A. Normal cells. B and C. Cells of *Proteus* grown in presence of penicillin. In C the cells are either swollen and misshapen, or "ghosts." In D the tendency to form long filaments is evident.

Salmonella. Gas not produced from sucrose, gelatin not liquefied. A considerable number of species are included here, many of them pathogenic.

The organisms which will be here considered are: *Salmonella enteritidis*, the cause of food poisoning, *S. paratyphi*, the cause of paratyphoid, *S. pullorum*, the cause of fowl salmonellosis and bacillary white diarrhea of chicks, and *S. typhosa*, the cause of typhoid in man.

In addition to these, *Salmonella cholerae-suis* is frequently associated with hog cholera as a secondary invader.

Classification of the Genus Salmonella. There is probably no other genus of bacteria on which as much work has been done from the standpoint of classification and differentiation. The complexities of the classification increasingly used are so great that specialists are required to understand or to name the various species, varieties, and groups. This has come about as follows. It was learned that bacteria belonging to this genus were the cause of many of the cases of food poisoning, so-called, and many cases of intestinal disorders of the type of mild dysenteries, etc. Isolations of the bacteria from victims in various outbreaks revealed that the bacteria differed in various respects, physiologically and particularly serologically. Many different species were described primarily on the basis of physiological characters such as fermentation of the several sugars. Later it was discovered that there were a great number of antigens present in these bacteria and it was proposed by White and Kauffman that all groupings and classification be on the basis of an antigenic analysis. It was believed that by this means positive identification of all strains might be achieved and real progress made in the study of outbreaks of disease and in recognition of their specific causes. There has been developed in consequence what is known as the White-Kauffman schema of antigenic analysis for identification. Because of the complexity of the materials and procedures, special laboratories have been set up in various parts of the world to serve as diagnostic centers. To these are sent cultures isolated for identification, or if new, for characterization.

In consideration of the serologic classification of the pneumococci it was pointed out that bacteria may have so-called somatic antigens and also capsular antigens for which antisera containing agglutinins may be prepared. The salmonellas may be shown to possess somatic antigens present in or on the body of the organism. These are named the "O" antigens and are relatively thermostable; they resist boiling in water. One kind of cell may possess one to several of these, which may be identified by the use of appropriate antisera. In addition, the salmonellas possess flagella which are also antigenic and for which antibodies may be prepared. The flagellar antigens are termed "H" antigens and are relatively thermolabile; they are destroyed by boiling. And to make the situation still more complicated, the same kind of organism when under different environment may show a different type of flagellar antigen. To the somatic antigens as they have been identified have been given Roman numerals. A series I, II, III . . . XXVII has been described. If the culture under examination is in phase I, its flagellar (H) antigens are designated by a small letter, if it is in phase II, by an arabic number. The complete antigenic formula for a species or variety of *Salmonella* consists of a series of Roman numerals, a series of lower case letters, and a series of arabic numerals. For example, the antigenic formula for *Salmonella cholerae-suis* has been worked out to be IV, V, XII: c : 1,3,4,5. It has three specific O antigens, one phase I H antigen, and four phase II H antigens. It is evident that by the use of this method of classification there will be all the pigeonholes needed for any number of organisms. Assuming that there may be a total of twelve O antigens, with no organism having more than five, and fifteen phase I H antigens with no organisms having more than five, and with five phase II antigens with no organism with more than five, there are possibilities of many thousands of antigenic formulae. Fortunately, things are not yet so complicated, for only about one hundred seventy-five different antigenic formulas have been developed thus far.

Very often when a new antigenic strain of *Salmonella* has been described it has been given a name based upon the locality where it was found, usually a city. We have, therefore, in the literature hundreds of names like *Salmonella london*, *S. newport*, and *S. arizona*. The authors of these names have not followed the recommendation of the International Rules of Nomenclature that specific epithets should be latinized. The names, however, are valid, as specific provision is made in the code for their recognition.

The present tendency is to recognize, as does the sixth edition of Bergey's *Manual*, a small number of species and to use the antigenic formulas and the other names for the designation of varieties.

The following classification will give the essential differences in the more important of the species recognized.

Key to Several Species of *Salmonella* Based on Physiological Characters

- a. Both acid and gas from glucose.
  - b. Acid from xylose. H_2S produced.
    - c. Acid from sorbitol.
      - d. Acid from inositol. Nitrates not reduced.
        Salmonella schottmuelleri
        S. typhimurium
      - 2d. No acid from inositol. Nitrates reduced.
        S. enteritidis
        S. hirschfeldii
        S. cholerae-suis
    - 2c. No acid from sorbitol.

2b. No acid from xylose. No H_2S produced. *S. paratyphi*
2a. Acid but no gas from glucose. *S. typhosa* (or *S. typhi*)

Salmonella enteritidis

Salmonella enteritidis may be taken as typical of a considerable number of species and varieties of organisms of the genus *Salmonella* associated with food poisonings. It was first isolated by Gaertner from the flesh of a diseased cow, a portion of which had been used for food and had given rise to fifty-seven cases of meat poisoning with one fatality. Since that time, this organism and closely related forms have been isolated from a great variety of sources, such as meats which have caused meat poisoning, from the feces, flesh, and blood of animals affected with various diseases, and even from the feces of healthy animals. It is perhaps the commonest cause of food poisoning, frequently mistermed ptomaine poisoning.

Morphology and Culture. This organism is an actively motile rod with rounded ends. It is about the size of the colon bacillus. Occasionally long filaments are observed resembling those found in cultures of the typhoid bacillus. It does not produce spores or capsules, is gram-negative, and stains well with ordinary aniline dyes. It grows best on ordinary alkaline media at blood heat, but well at room temperature. It is aerobic and facultative anaerobic. Broth is rendered turbid. Gelatin is not liquefied. Its growth in milk is most characteristic. At the end of a day very little change is to be seen, though the reaction becomes weakly acid. After a long period, from one to several weeks, the milk becomes yellowish and somewhat transparent, and a strong alkaline reaction is developed. The milk is never coagulated. Indole is sometimes produced in small quantities in peptone broth, but the reaction is usually uncertain. Lactose and saccharose are never fermented, but dextrose is fermented with the development of both acid and gas. This organism is somewhat more resistant to heat than are the other members of the intestinal group. It has been found that 60° C. for an hour is necessary to destroy it, and for shorter periods higher temperatures are required. It has also been found to be more resistant than most related bacteria to the action of the antiseptics used in smoking and pickling foods.

Disease Production. The smaller laboratory animals all succumb to subcutaneous, intravenous, and intraperitoneal injections and to feeding of this organism. The most susceptible animal is the mouse. Some strains of this organism have also been found capable of producing disease in calves, swine, monkeys, dogs, and cats. However, the virulence of the various strains which have been isolated is exceedingly variable.

The production of disease by this organism when taken into the intestinal tract of man is probably to be ascribed to two causes. The first is the development by the organism, while growing in the food itself, of an unusually soluble and powerful endotoxin differentiated from true toxin in that it is exceptionally heat-resistant. Even the temperature of boiling water does not greatly reduce its potency. Meat which has been infected and quite thoroughly cooked may give rise to this form of poisoning even though all the bacteria have been destroyed. The quick development of symptoms in persons eating such food is probably due to this endotoxin. Second, this organism may multiply in the intestine or its wall and produce a disease resembling in some respects typhoid fever. This is fatal in a small proportion of cases.

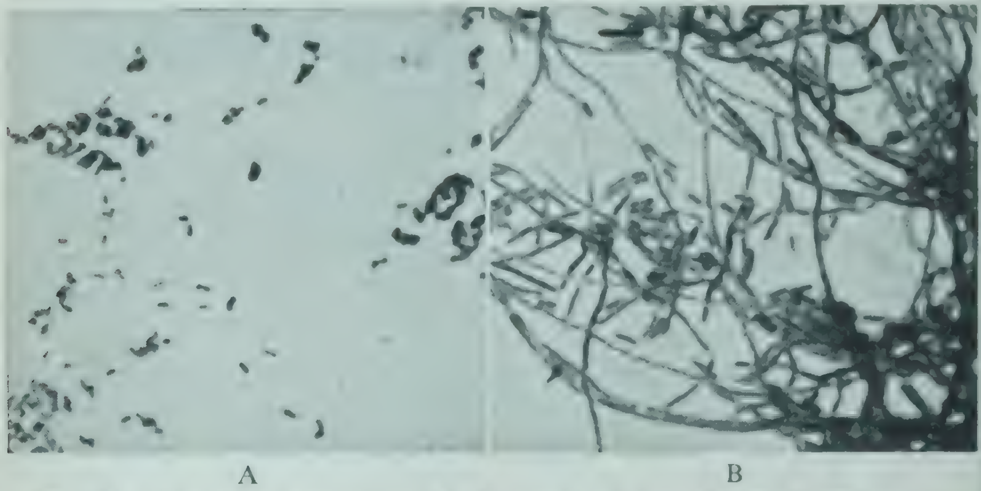

Fig. 34–2. *Salmonella enteritidis.* A. Normal rod-shaped cells. B. Filamentous cells which develop when grown in presence of penicillin.

Some of the so-called "viruses" formerly sold on the market for the purpose of exterminating rats and mice are probably cultures of this organism or a closely related species. These have been exploited as capable of causing a fatal disease in these rodents, but not pathogenic for man or any of the higher animals. However, in some cases at least, tests have shown that these organisms are capable of infecting higher animals and even man. There does not seem to be any good reason for differentiating this rat "virus" from *S. enteritidis.*

Very great differences in virulence have been noted in strains isolated from different sources. It is probable that continued residence and growth in a single species of animal may develop an unusually high disease-producing power relative to that particular species, while pathogenicity for some other species may not be increased. This may account for the very considerable number of distinct strains and varieties of this organism that have been isolated. In many cases these are believed to be distinct species.

Immunity. No practicable method of immunization of man against this disease has been developed or practiced. Agglutinins specific for the organism causing an infection are developed in the blood and may be used in the diagnosis of the disease. The poisonous product of the growth of this organism is, as has been noted above, an endotoxin and not a true toxin, hence no antitoxins have been developed.

Transmission. This organism is probably the most important cause of the so-called meat poisoning, also incorrectly known as ptomaine poisoning. Infection of flesh may be either antemortem or postmortem. Antemortem infection may be the result of either primary or secondary infection. The organism is present in the blood stream and in the tissues in a diseased animal and probably continues to multiply after its death. It can also gain entrance to flesh after the animal has been slaughtered. It has been found in the normal feces. Its presence in such is one of the chief arguments for cleanliness about slaughterhouses. During the slaughtering of animals and preparation of their flesh for the market, every precaution should be used to see not only that the animal is not diseased, but also that fecal material is never allowed to come in contact with the healthy tissues. This means the rigid exclusion of flies and a high degree of cleanliness in all the operations. Experiments have shown that when *S. enteritidis* is placed upon the surface of fresh meat, it rapidly penetrates to the interior of the tissues even when the meat is stored at a relatively low temperature.

Salmonella paratyphi

Synonyms. What has been said relative to the synonymy of *Salmonella enteritidis* is even more true of the synonymy of this organism.

Organisms of this type have been repeatedly isolated from cases of so-called paratyphoid fever in man, a disease resembling clinically the true typhoid fever but differing in that the blood serum does not give the characteristic agglutination reaction with typhoid bacilli, but only with the organism isolated from the feces of the particular individual. (This organism has likewise been isolated from many other sources as noted above.)

Morphology and Culture. These characters do not differ in any marked degree from those noted for the preceding organism *S. enteritidis*.

Disease Production. Paratyphoid fever in man is frequently diagnosed as the result of food infection. In some cases it has been definitely shown to be due to the use of contaminated water and milk. In the majority of cases it is probably due to the consumption of infected meat. The disease in man has been attended by a low mortality. It manifests itself as an acute inflammation of the intestines.

Salmonella pullorum

This organism is the cause of bacillary white diarrhea in chicks (salmonellosis or "*pullorum*"[1] disease of fowls), one of the most serious diseases of poultry and one which causes great economic losses. Two strains of organisms are known; one, typical, which produces gas, and an anaerogenic strain, which does not. Morphologically and culturally this organism is closely related to the other members of the genus.

The disease occurs in young chicks, causing a white diarrhea, and a "pasting up" which is quite characteristic. The birds become emaciated and usually succumb, although a few recover. The organisms are excreted in large numbers, and the disease is transmitted from bird to bird by ingestion.

Of special interest is the fact that certain hens are carriers of the infection. The organisms are present in the ovary and present also in the eggs when laid. Chicks hatched from such eggs already are infected and soon show symptoms of the disease. From these chicks the disease rapidly spreads to others. It is evident that the elimination of carriers from flocks of hens is most important.

Carriers may usually be recognized by means of the agglutination test. In valuable flocks it is possible to eliminate the carriers and thereby eliminate the disease by its use.

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[1] *Pullorum* is a Latin plural genitive meaning "of chicks." Pullorum disease would mean "disease of chicks." "Pullorum disease of fowls" means literally "chicks' disease of fowls," certainly a highly undescriptive term. *Salmonellosis* is to be preferred.

SUBGROUP III. TYPHOID-DYSENTERY SUBGROUP

This subgroup includes those enterobacteria which do not produce gas from carbohydrates. Two principal genera have been recognized, *Eberthella* and *Shigella,* the species of the former motile and containing the typhoid bacteria, those of the latter including the dysentery bacteria. With the development of the White-Kauffman schema it was found that the typhoid bacillus has some common antigens with species of the genus *Salmonella,* and so, in spite of physiologic differences, the typhoid bacillus has been transferred by the advocates of this schema to the genus *Salmonella.* The sixth edition of Bergey recognizes this transfer, and will be followed here. There is some question as to the ultimate general acceptance of this change. There is involved the whole question as to whether serologic resemblances or physiologic resemblances should be regarded as better criteria in classification.

Salmonella typhosa

Synonyms. *Bacillus typhi-abdominalis, Eberthella typhosa, Eberthella typhi, Salmonella typhi, Bacillus typhosus, Bacterium typhosum,* Antigenic formula IX, XII: d:

There is some question as to whether the specific epithet should be *typhosa* or *typhi.* Historically, two diseases having certain clinical resemblances were named *typhus abdominalis* and *typhus exanthematici.* The former has come to be called typhoid in America and enteric fever in England. The latter is commonly termed typhus. The organisms causing the two diseases are not related; typhoid is caused by a true bacterium, typhus by rickettsias.

The organism was first noted by Eberth, who found it in the spleens of persons who died of typhoid fever. It was first cultivated in 1884 by Gaffky. It is difficult, if not impossible, to produce typical typhoid fever in the usual laboratory animals.

This disease is widely spread in temperate and tropical countries and is constantly present in the United States. It is one of the most important diseases with which the sanitarian has to contend.

Morphology and Culture. *Salmonella typhosa* is a plump rod 0.5–0.8 μ by 1.0–3.0 μ. Usually it occurs singly, occasionally in short chains, sometimes producing long filaments probably to be regarded as involution forms. It is actively motile by means of numerous peritrichous flagella. It does not produce capsules or spores, it stains readily with aniline dyes, and is gram-negative.

The organism may be isolated from the blood or spleen of an individual having typhoid fever, and by means of appropriate culture media it may be obtained from the feces. It is exceedingly difficult, however, to detect it in water. This has been accomplished only a few times. The colonies upon gelatin are smaller and more delicate than those of *Escherichia coli*. It grows best at a temperature of 37° C., but will develop at room temperature. It is aerobic, but becomes facultative anaerobic in the presence of sugars which it can ferment, such as dextrose. It does not coagulate milk or digest casein.

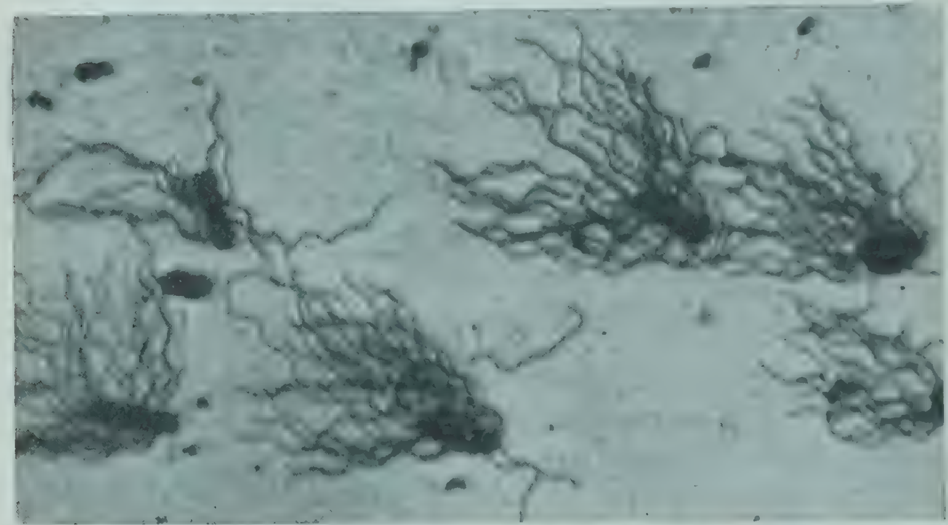

Fig. 34–3. *Salmonella typhosa.* Photograph of stained preparation showing the numerous peritrichous flagella. (Courtesy General Biological Supply House, Chicago.)

Disease Production. Typhoid as a disease in the United States and in some other countries is far less prevalent than formerly. It has been estimated that in the period about 1900, about one person in two hundred contracted this disease each year. Five decades later, the disease has practically disappeared. In seventy-eight cities which maintained adequate records the death rate per 100,000 population decreased between 1910 and 1942 from 20.5 to 0.26, a reduction of more than 98 per cent. The injection or inoculation of the typhoid bacillus into laboratory animals, such as the guinea pig, produces results differing but little from those obtained by the injection of *Escherichia* *coli*. By means of feeding experiments, it has been found possible to produce the disease in certain monkeys. Accidental infection of man has also occurred in the laboratory from pure cultures. There is no reason for doubt, therefore, as to the causal relationship of this organism to the specific disease. It gains entrance to the body through the alimentary tract, probably multiplies to some extent in the intestinal contents, and invades the lymph system, particularly the clusters of lymph glands called Peyer's patches of the small intestines. These latter become ulcerated and the hemorrhages characteristic of this disease and the occasional intestinal perforations are due to these ulcerations. The organism also invades the blood stream so that the disease is to be regarded as a true bacteremia. It is present in very great numbers in the spleen. This latter organ becomes considerably swollen. Small "rose spots" usually are produced on the skin of the abdomen. Occasionally, even after recovery, the infection may persist in some part of the body, as in the gall bladder or in the bones or joints. The organism does not produce a true toxin.

Immunity. An individual convalescing from typhoid shows a considerable degree of immunity, but this disappears gradually, and the disease may again attack the same person. Antisera have been prepared and tried, but have not proved successful. Within recent years vaccination against typhoid fever has become relatively common. The vaccine is prepared by growing the bacilli on the surface of agar slants, scraping them from the surface of these cultures and suspending them in physiological salt solution, heating to a temperature high enough to destroy the organism. The vaccine is standardized to a content of one billion bacteria per milliliter. Three injections are usually made, spaced at weekly intervals, the first of 500 million and the second and third of one billion bacterial cells. These vaccines have been extensively used for the prevention of typhoid in the armies. They are, in practice, frequently combined with bacterins prepared from cultures of paratyphoid bacteria.

Agglutinins develop in the blood of those who have typhoid fever, and use is made of this fact in carrying out the Widal or agglutination test in the diagnosis of the disease. Sometimes the organisms are cultivated directly from the blood of the patient and the disease is thus diagnosed.

Transmission. Typhoid fever is frequently contracted through drinking water. A city which has a polluted water supply practically always has a high typhoid death rate. The effort made by sanitary engineers and others for the development of suitable water supplies has largely been for the purpose of stamping out this disease.

Milk and other foods may also serve as carriers of this organism. Milk is contaminated only after being drawn from the animal, as the cow itself does not contract typhoid fever. The organism may gain entrance to the milk through the use of polluted water in washing milk vessels or by contact with a person having the disease. It is probable that flies are frequently instrumental in carrying the typhoid bacillus from the dejecta of typhoid patients to food. Within recent years a determined effort has been made to brand the common house fly as the "typhoid fly," inasmuch as it breeds in garbage piles and excreta and undoubtedly is a common carrier of the bacteria.

Direct infection or contact infection is also relatively common. All the excretions of the typhoid patient should be carefully disinfected, and care should be used to see that all dishes and utensils used by the patient are boiled.

A very important factor in the spread of typhoid fever is the so-called carrier. This term is used to indicate an individual who continues to harbor a pathogenic organism after apparent recovery from the disease. The bacteria continue to be passed off with the body excretions for a variable period during convalescence, sometimes for eight to ten weeks after the onset of the disease. These are convalescent carriers. However, some individuals become chronic carriers. Typhoid bacilli have been found in the excretions of individuals that have had typhoid fever several years previous to the examination. Such individuals are a real menace to the health of a community, as it is very difficult indeed to guard others from infection. Fortunately only a small number of those who have typhoid fever continue to carry the organisms long after convalescence. Occasionally an individual may have a mild case or a case of walking typhoid. In such instances, evidently the body is sufficiently resistant to prevent the disease from running a typical course, but such individuals are just as dangerous to others as those who have a severe or even fatal case of the disease.

It may be emphasized that typhoid bacilli do not retain their vitality for a long period outside of the body. There is no evidence that they ever multiply in filth or dirt of any kind. They certainly are never carried by air currents. They leave the body only in the excretions, and the most common way of contracting typhoid is to drink liquids or eat foods which have been contaminated from such excretions directly or indirectly.

Shigella dysenteriae

This is the cause of bacillary dysentery in man. The organism was first discovered in 1898 by Shiga in Japan. In 1900 Flexner described a somewhat similar organism in the Philippine Islands. Since that time dysentery has been carefully studied and a number of types of organisms have been isolated, making necessary the differentiation of several species. The bacillus of Shiga is probably the most common and the most important, but the bacillus described by Flexner has also been found in a certain proportion of cases.

Morphology and Culture. The dysentery bacillus resembles the typhoid bacillus in stained mounts but has no flagella. It stains readily with aniline dyes and is gram-negative. Cultural characters are very similar to those of *Salmonella typhosa*. Milk is rendered permanently alkaline. The species and varieties of *Shigella* that have been differentiated have been separated on the basis of carbohydrate fermentation. As many as fifteen different groups have been created by this method by some authors. The bacillary dysenteries are not to be confused with the dysentery caused by an ameba.

Disease Production. As in typhoid fever, the organism gains entrance to the body by way of the alimentary tract. The intestines, particularly the colon, are inflamed. In some cases the latter may even show necrosis of the lining membranes. The organism does not usually gain entrance to the blood and is not common in the internal organs, with the exception of the lymph glands adjacent to the intestines. It is probable that the disease is a toxemia rather than a bacteremia.

Immunity. It has been found that the Shiga type of dysentery bacillus produces a toxin which is probably responsible for the direct injury to the intestinal walls. The toxin has been prepared in the laboratory by growing the organism in an alkaline broth, and the antitoxin by the systematic injection of such broth into suitable animals. This antitoxin has been found to give favorable results in the treatment and cure of the disease caused by this type of organism. The other types of dysentery bacilli do not seem to produce a true toxin, and no efficient method of treatment by the use of an antiserum has thus far been perfected.

The blood serum of an individual infected with dysentery is found to contain agglutinins specific for the particular type of organism causing the disease. The agglutination reaction has been the principal means of differentiation of the various types of organisms. It enables one, by testing the blood serum with each of the types of dysentery bacilli, to determine with a fair degree of accuracy the particular type causing the disease in the individual under examination.

Transmission. Dysentery is transmitted in the same manner as typhoid fever, that is, by water, milk, food, and by direct contact. There is no evidence that the diarrheas and dysenteries of the lower animals are ever produced by these bacteria. Probably carriers are even more important in the transmission of this disease than in typhoid.

CHAPTER 35

Undulant Fever—Abortion Group: The Genus *Brucella*

Organisms causing three diseases are to be placed here. These diseases are undulant fever in man, caused by *Brucella melitensis:* bovine infectious abortion caused by *Brucella abortus*[1] and brucellosis of swine caused by *Brucella suis*. These organisms are very closely related, in fact it may be questioned whether they should be grouped together as a single species with three varieties or be recognized as separate species.

The *Brucella melitensis* was named by its discoverer Bruce (1887) from the Latin name of the island of Malta where it was first isolated. He regarded it as a coccus and named it *Micrococcus melitensis*. Later Bang (1895) described the organism now known as *Brucella abortus* from the aborted fetus of a cow. Traum (1914) was the discoverer of a disease now known as brucellosis of swine. That these three organisms were closely related was not known until the classic work of Alice Evans, who studied cultures from a number of sources.

Brucella melitensis

Synonyms. *Micrococcus melitensis, Alcaligenes melitensis, Bacterium melitense.*

This organism is the cause of the disease variously called Malta fever, Mediterranean fever, or undulant fever. It is known from southern Europe and northern Africa, from Mexico, and other countries. In the southwestern United States it is known as goat herders' disease.

Morphology and Culture. *Brucella melitensis* is a short non-motile bacillus, often almost spherical. It is about 0.5 μ in diameter and occurs singly or in short chains. It is gram-negative and does not produce spores. Enriched media at pH of about 6.8 such as liver infusion agar, liver broth, or tryptose agar are useful in securing growth. It may be differentiated from the other species by the fact that addition of CO_2 is not required for growth, it produces little or no H_2S and will grow in a medium having a concentration of 1–50,000 of the dye thionine or 1–25,000 basic fuchsin.

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[1] Note that the specific epithet *abortus* is a Latin noun in the genitive and is in correct form and means *of abortion*.

Disease Production. The symptoms of the disease in man tend to persist for a relatively long period. There is an undulating fever, severe articular rheumatism and extreme sweating, particularly at night. Although not highly fatal, the disease is a very serious one due to its long course and the extreme debilitation produced.

Diagnosis of the disease is sometimes accomplished by isolation of the causal organism from the blood. The agglutination test may be used, but is not highly reliable inasmuch as the blood of uninfected individuals may contain agglutinins.

Transmission. The disease primarily affects the goat, and is most frequently acquired by drinking the unpasteurized milk from infected goats. The peculiar incidence of the disease may in part be explained by the distribution of goats. It has also been contracted on several occasions by laboratory workers who were handling cultures of the organism or working with infected laboratory animals as guinea pigs. It is of special interest in the United States because of its relationship to the organism next to be considered.

Brucella abortus

Synonyms. *Bacillus abortus, Bacterium abortus, Alcaligenes abortus.*

The organism which causes bovine infectious abortion was first described in Denmark by Bang in 1897. The disease is very widespread in Europe and in America and is one of the most important affecting the live stock industry.

In general the morphologic and physiologic characters of *Brucella abortus* resemble closely those of *Br. melitensis* except that CO_2 is needed for growth. Isolation is best in an atmosphere containing about 25 per cent CO_2. Proof of very close relationship was developed by Alice Evans. The organism may be differentiated from the other species in that some H_2S is produced in culture, growth occurs in medium containing basic fuchsin 1–25,000, but not in thionine 1–50,000.

Disease Production. In the cow infection leads to an inflammation of the mucous membrane of the uterus with consequent abortion if the animal is pregnant. A cow which has aborted once or twice may develop an immunity and thereafter carry the calf to full term. The abortion disease has been very troublesome in dairy herds and has led to serious economic losses.

It has been found possible to infect the guinea pig, with consequent development of a characteristic arthritis.

Of particular interest is the relationship of the abortion bacillus to disease in man. There have been numerous cases of undulant fever reported in which there was no probability of infection from the goat and in which the organism differed in minor respects, particularly immunologically, from the true *Br. melitensis*. From these cases typical *Br. abortus* cultures have been isolated.

Brucella suis

This was first isolated in 1914 by Traum from brucellosis in swine. In morphology and culture the organism differs but little from the other two species. In does not require CO_2 for growth, produces H_2S rapidly, and grows in broth with thiamin 1–50,000 but not with basic fuchsin.

The disease in swine frequently produces abortion, and often arthritis, the animal becoming lame. The disease is usually diagnosed by use of the agglutination test.

Of special significance is the fact that a large proportion of cases of brucellosis in man may be due to infection with *Br. suis*. It has been frequently reported in those who handle pork in meat packing establishments.

CHAPTER 36

The Cholera—Pseudomonad Group: The Genera *Vibrio* and *Pseudomonas*

The organisms of this group are similar in being rods motile by means of one to a few polar flagella. The members of the genus *Vibrio* have cells that are slightly bent to sharply curved, in *Pseudomonas* the cells are straight. Only three representative species will be discussed. *Vibrio comma* (*cholerae*) the cause of Asiatic cholera in man, *Vibrio fetus, a* cause of abortion in sheep and cattle, and *Pseudomonas aeruginosa,* the blue pus organism.

Vibrio comma

Synonyms. *Spirillum cholerae, Spirillum cholerae asiaticae, Microspira comma.*

This organism is the specific cause of Asiatic cholera in man. It was first described in 1883 by Koch, who found it in great numbers in the "rice-water" stools of cholera patients. The disease is constantly present in certain parts of India and in China, and on several occasions has spread in epidemic form to other parts of the civilized world, particularly to Europe. The chief endemic focus seems to be located in lower Burma. There have been several epidemics in the United States, but none since 1900.

Morphology and Culture. The Asiatic cholera vibrio is a slightly curved rod. Filamentous and involution forms are not infrequently observed in culture media. A single polar flagellum is present. Spores and capsules are not formed. It is gram-negative and stains readily with the ordinary aniline dyes. Various methods of isolation of the organism have been developed. It is not difficult to secure the organism in pure cultures from the feces of cholera patients by plating upon nutrient gelatin. The organism will grow in a medium which is distinctly alkaline, with a pH of 8.5–9. Numerous selective media have been developed based upon this characteristic as well as the fact that when grown in broth it shows marked aerotaxis and tends to collect at the air-liquid interface. The organism is aerobic and grows readily at room temperatures, although the optimum is blood heat. It is easily destroyed by high temperatures, 60° C. for a few minutes being sufficient. It is also easily destroyed by drying, by disinfectants, and by sunlight. It liquefies blood serum and gelatin. Milk is not coagulated.

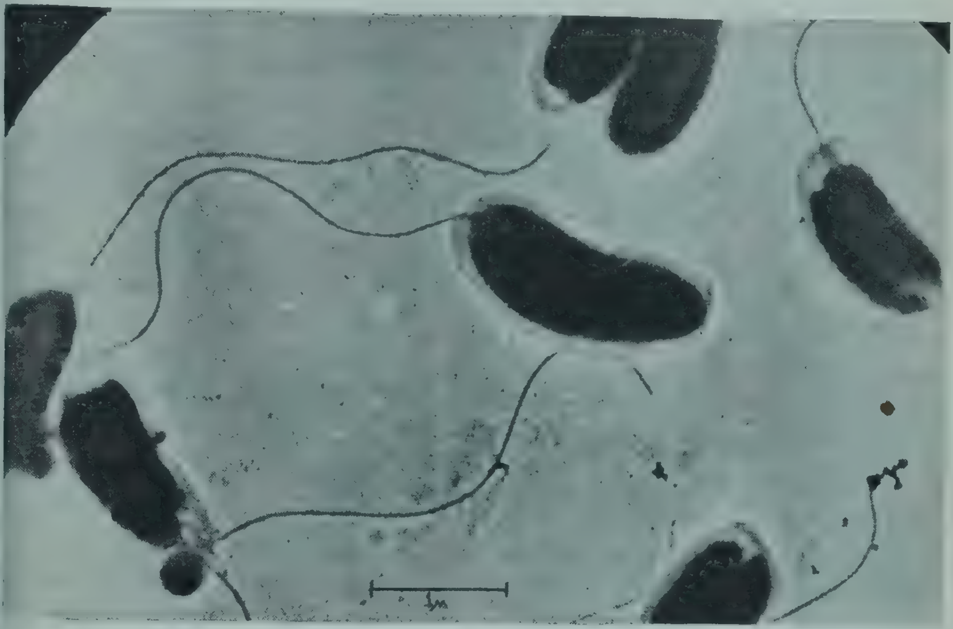

Fig. 36–1. *Vibrio comma.* Electronograph. Note that the protoplasm has shrunken away from the outer cell wall, showing the surface of the plasma membrane. Each cell has a polar flagellum, which apparently penetrates the cell wall and is continuous with the protoplasm of the cell. (Courtesy of G. Knaysi and S. Mudd and the *Journal of Bacteriology*.

Disease Production. The injection of cultures of the Asiatic cholera organism into laboratory animals in sufficient quantities usually proves fatal, but the disease produced is not typical Asiatic cholera. The disease as it occurs in man manifests itself as a severe type of diarrhea. The organism produces poisons which cause necrosis of a portion of the intestinal epithelium which sloughs off, giving rise to the so-called rice-water stools, the fragments of epithelium resembling particles of rice.

Immunity. Agglutinins are developed in the blood of individuals having Asiatic cholera, but not early enough to be of great value in diagnosis. The blood of individuals that recover from the disease and that of animals that are artificially immunized contain bacteriolysins. It is possible that immunity to this disease is largely due to these substances. Vaccination has been extensively practiced, particularly in India. The vaccine consists of cultures that have been killed by exposure to a temperature of 58° C., or in some cases of cultures attenuated by growth at temperatures somewhat higher than their optimum.

Transmission. This disease is generally transmitted through impure water and food. In Asiatic countries, as China, where night soil is commonly used in fertilization, the vegetables may sometimes be infective. Carriers are of importance in this disease. During 1911 a number of cases of Asiatic cholera bacillus carriers were identified, by means of culture methods at the port of New York, among immigrants. Such individuals might easily serve as foci for an epidemic of the disease.

Vibrio fetus

Synonym. *Spirillum fetus.*

This organism was first observed by MacFadyean and Stockman (1909) as the probable cause of abortion in ewes. Theobald Smith (1918) isolated it from aborting cows, and it was named a year later by Smith and Taylor (1919).

The cells are somewhat pleomorphic, in young cultures short, in old cultures elongate spirals: 0.2–0.5 μ by 1.5–5.0 μ.

Motile by means of a single flagellum, sometimes two. Granules may be stained in the cells. The organism is gram-negative.

The organism is somewhat difficult to isolate and to secure growth in laboratory media. Usually a medium containing blood or fresh tissue is required, and growth is favored by the presence of carbon dioxide. Isolations may be made upon an agar slant enriched as indicated. The growth is delicate at first. Very small colonies one to three millimeters in diameter and somewhat raised above the surface form on blood agar. It does not ferment carbohydrates. It is nonpathogenic for laboratory animals.

In ruminants it invades the uterine membranes and produces abortion.

Pseudomonas aeruginosa

Synonyms. *Bacterium aeruginosum, Bacillus pyocyaneus, Pseudomonas pyocyanea.*

That purulent exudates may produce a bluish green stain o surgical dressings was known some years before Schroeter (1872) named the causal organism *Bacterium aeruginosum*.[1] It was renamed *Bacillus pyocyaneus* and studied extensively by Gessard (1882 and later).

Morphology and Culture. The cells are usually slender rods 0.5 μ by 1.5–3 μ. They may be united in short chains. They are actively motile by means of one to three polar flagella. No capsules are produced. The cells stain readily and are gram-negative.

This pseudomonad grows readily upon most culture media. On nutrient agar the colonies tend to be large and to spread; they are butyrous (buttery) in consistency. Growth is good at room temperature, better at blood heat. The organism is aerobic. It liquefies gelatin rapidly and produces hydrogen sulfide in a favorable medium. In litmus milk a soft coagulum is produced followed by rapid liquefaction of the curd and by digestion. The reaction becomes alkaline. There is luxuriant growth on potato. Some acid may be produced from glucose, but other sugars are not acidified.

Particularly noteworthy is the production in most cultures of pigment which diffuses through the medium. The color is usually green or blue-green, in old cultures becoming brownish. At least two pigments are produced. A pigmented broth culture may be extracted with chloroform which dissolves the blue pigment *pyocyanin*. The production of pyocyanin is characteristic of *Pseudomonas aeruginosa*. The second pigment, a *fluorescein* is soluble in water but not in chloroform. It is fluorescent[2] and yellowish green in color. The latter pigment is found also in other species of *Pseudomonas*.

Disease Production. *Pseudomonas aeruginosa* is associated with disease condition both in man and animals, possibly also in plants under the name *Pseudomonas polycolor*. It may occur in infected wounds, then commonly in association with other bacteria. In man it may cause abscesses, perhaps most commonly infections of the middle ear. It has been isolated from cases of endocarditis, pneumonia, and even dysentery-like disease.

In domestic animals it is not uncommon in wound infections. It is characteristically present in "bull nose" of the pig.

When injected intraperitoneally into the guinea pig it produces death in twenty-four to thirty-six hours.

[1] From the Latin *aeruginosus* meaning the blue-green associated with verdigris or oxidized copper.

[2] Fluorescent means that the pigment appears to be one color when viewed by transmitted light and another color viewed by reflected light.

The organism is of interest because from its cultures the first antibiotic *pyocyanase* was prepared. It has been much studied. It is quite active in the destruction of other microorganisms, such as certain cocci and the causal organisms of diphtheria, cholera, and plague. While the compound has been used successfully therapeutically, it has never come into general use and has been displaced by other antibiotics.

CHAPTER 37

Group of Plant Pathogens: The Genera *Pseudomonas*, *Xanthomonas*, *Erwinia*, *Corynebacterium*

The bacteria known to produce plant diseases are of many kinds. They will not be considered in detail since this would require a volume in itself. The genera in which these bacterial species are found and a few of the more important species may be noted.

Burrill (1882) was apparently the first person definitely to associate a bacterium with a specific plant disease. He studied the important disease fire blight of the pear and apple and named the organism *Micrococcus amylovorus,* now the type species of the genus *Erwinia,* and known as *Erwinia amylovora.* The next year Wakker in Holland described an organism causing a yellow rot of the bulbs of hyacinth as *Bacterium hyacinthi.* It is now considered to be a species of *Xanthomonas.* The species now known as *Xanthomonas campestris* described by Pammel (1895) as the cause of the disease black rot of cabbage was one of those most studied in the early days of work with bacterial plant diseases. There was a school of thought headed by Fischer in Germany which contended that bacteria did not produce disease in plants, that when and where they were found in plants they were secondary invaders. Dr. Erwin F. Smith of the United States Department of Agriculture took up the challenge and debated the problem of the relationship of bacteria to plant disease in the scientific press. He definitely proved his point, and in the proving gave a great impetus to the study of the bacteria pathogenic for plants. It was found that the postulates of Koch could be applied as satisfactorily to plant pathogens as to animal pathogens.

The diseases caused by bacteria in plants are usually of one of five types. They are the soft rots, wilts, blights, galls, and spots. Soft rots are caused by microorganisms that produce pectolytic enzymes that dissolve the pectic substances which bind cells together, particularly in the softer tissues. This leads to a falling apart of the cells and to their destruction, the tissue becoming a mass of soft rot. In the wilts the organisms invade the vascular system of the plant and prevent the flow of water or of nutrients, resulting in the desiccation of the tissues supplied. The blights in some measure resemble the wilts. The organisms penetrate through the intercellular spaces and vessels and cause the adjacent cells to die. The galls are produced by organisms that cause the plant to develop new tissue rapidly at the point of invasion, leading to the formation of plant tumors, which in some respects are the plant analogs of cancer. In the spots the bacteria remain localized and kill but a relatively small area of tissue.

In addition to the plant diseases here to be considered as caused by true bacteria there are others produced by ray bacteria (*Streptomyces*), protozoa, and viruses. These will be noted later.

The nomenclature of the bacteria that cause plant disease is not in a very satisfactory state. Most of the bacteria belong apparently to one of four genera, *Pseudomonas, Xanthomonas, Erwinia,* and *Corynebacterium.* There are some important organisms that are not allocated in the last edition of Bergey's *Manual* to any genus but are placed in the catch-all grouping of *Bacterium.*

Species of Pseudomonas Producing Plant Diseases

The genus *Pseudomonas* has previously been defined as including gram-negative rods, motile by means of one or few polar flagella, and producing generally a diffusible pigment, usually greenish in color, though other colors may be produced, and some species produce no pigment. The principal species parasitic on man and animals, *Pseudomonas aeruginosa,* has already been described. Members of this genus are widely distributed in soil and water. Several plant diseases have been described as caused by pseudomonads. Burkholder in the sixth edition of the Bergey *Manual* recognizes eighty-four species, with the notation that there are many more insufficiently described to warrant acceptance. Of these, fifty-eight produce the typical fluorescent pigment, twenty-six do not. Most of the species described are of little economic significance. Among those occurring on crops of importance are *Pseudomonas striafaciens* which produces a streak in the leaf blade of the oat, *P. angulata,* from angular leaf spot of tobacco, *P. coronafaciens* from halo spot in the leaves of oats, *P. lachrymans* pathogenic on cucumber and other cucurbits, *P. medi-*

caginis producing brown lesions on leaf and stems of alfalfa, *P. phaseolicola* causing the halo blight of beans, *P. pisi* producing water-soaked lesions on the stems of the pea, *P. atrofaciens* causing a basal glume rot of wheat, *P. apii* from diseased celery leaves, *P. mors-prunorum* producing cankers on plum, *P. tabaci* from wild fire lesions of tobacco, *P. setariae* causing brown stripe on Italian millet, *P. polycolor* from tobacco leaf spot (this organism is very like *P. aeruginosa,* pathogenic for man, possibly identical), *P. viridiflava* from the bean, *P. tolaasii* causing a disease of the cultivated mushroom, *P. xanthochlora* from rotting potato tubers, *P. barkeri* from blossom blight of the pear, *P. glycinea* from the soybean, *P. savastanoi* from the galls of the olive, *P. alliicola* from rot of the bulb of the onion, *P. solanacearum* attacking plants belonging to the family *Solanaceae* such as potato, tomato, and tobacco, *P. radiciperda* causing root rot of legumes, particularly alfalfa, *P. andropogoni* from the sorghum leaves and stems, and *P. wieringae,* pathogenic for beets.

Some of these bacteria such as the one causing the root rot of alfalfa are of considerable economic significance. The disease constitutes one of the major problems in the growing of alfalfa in the Mississippi Valley, and an extensive program of plant breeding is under way in order to find varieties that are resistant.

Emphasis should be laid on the fact that for most of the diseases that are produced by bacteria and that affect plants the most feasible remedy is the breeding and development of plants that are relatively immune. Much progress has been made in this direction.

Species of Xanthomonas Producing Plant Disease

The genus *Xanthomonas* is very closely allied to *Pseudomonas* and, in fact, was created by splitting the genus *Pseudomonas* into two. The xanthomonads do not produce a green diffusible pigment, and the colonies on suitable media, as nutrient agar, are usually some shade of yellow or brown. The genus was created initially to include a considerable group of bacteria pathogenic for plants though there are also some purely saprophytic species. The cells are monotrichous.

Proteins such as casein are readily digested, and milk usually is rendered alkaline. Acid is usually produced from the monosaccharides and the disaccharides. The numerous species that have been described for the most part produce necrosis of plant tissues.

Burkholder in the sixth edition of the Bergey *Manual* recognizes forty-seven species as pathogenic for plants. It is probable that with time some of these species will be united or recognized as varieties having special adaptation to a particular host. Some produce diseases that are economically important. A few of these may be listed.

The type species, *Xanthomonas hyacinthi*, produces a rot in hyacinth bulbs; *X. pruni* was isolated from diseased plums; *X. vitians* produces a stem disease of lettuce; *X. rubrilineans* is found in the reddened stripes in the leaves of the sugar cane; *X. campestris* causes the serious disease black rot of cabbage and of many other cruciferous plants; *X. citri* produces galls on citrus trees; *X. cucurbitae* is from a leaf spot of the squash; *X. juglandis* causes black spot of leaves and fruit of the English walnut; *X. malvacearum* produces the angular leaf spot, an important disease of cotton; *X. phaseoli* causes pustules on the leaves of the bean and of the soybean; *X. translucens* is from the barley leaf spot; *X. vasculorum* causes gummosis of the sugar cane; *X. gummisudans* is from gummy lesions of the leaves of gladiolus, *X. oryzae* from a leaf blight of rice, and *X. rubrisubalbicans* from mottled stripe of the leaves of sugar cane.

Species of Erwinia Producing Plant Diseases

The genus *Erwinia* is the single genus of the tribe *Erwinieae* included in the family *Enterobacteriaceae*. The tribe and genus were created to care for the plant pathogens which physiologically, morphologically, and culturally seem to belong with the intestinal group of bacteria. There is some question whether plant pathogenicity alone should constitute the sole criterion for the recognition of this genus. Obviously a culture of any one of the species could not be identified if the fact that it was a plant parasite was unknown. However, the genus serves a useful purpose with its present definition until a better one can be devised.

The bacteria belonging to the genus *Erwinia* are rods, usually motile by means of peritrichous flagella. All are gram-negative: all are able to produce acid from one or more sugars, some species form gas also. Gelatin is liquefied by some species and not by others. All are plant pathogens producing several types of lesions, a dry necrosis, galls, wilts, or soft rots.

From the standpoint of the lesions produced in the host plants the genus can be divided sharply into two groups. The first includes those species which cause lesions other than soft rots, such as dry necroses, galls, or wilts. This group contains the type species, *Erwinia amylovora*, the cause of pear blight and the first proved bacterial plant parasite. The second group contains those species which produce the soft rots. The bacteria invade tissues, usually those which are succulent, producing an enzyme which digests the pectin (protopectin) of the middle lamella releasing the cells and permitting them to fall apart, causing complete disintegration, hence the designation, soft rot. Waldee (1945) proposed that these soft rot organisms be placed in a separate genus which he appropriately named *Pectobacterium*.

Burkholder, in Bergey's *Manual* (6th ed.) recognizes six species in *Erwinia* exclusive of the soft rot species. Of these, two are of importance in the United States: *Erwinia amylovora* causes the serious disease bacterial blight of pears and attacks more or less seriously many species of plants belonging to the rose family (*Rosaceae*), including the apple.

Among the *Erwiniae* producing soft rots (Waldee's *Pectobacterium*) sixteen species are included. The best known of these, *E. carotovora* (*Pectobacterium carotovorum*), is probably the most important, producing soft rots in a large number of plants, including carrot, cabbage, celery, cucumber, eggplant, iris, muskmelon, hyacinth, onion, parsnip, radish, and tomato. Different names have been given by various authors to this organism as found in the various hosts; thirteen such are listed in Bergey. This organism produces acid and gas from many sugars, including lactose. Its cultures are methyl red positive and Voges-Proskauer negative, resembling *Escherichia coli* except for its ability to liquefy gelatin and to produce soft rot.

Another interesting soft rot is that caused by *Erwinia carnegieana* in the giant cactus of the southwestern United States. Black stem and tuber rot of potato is caused by *E. atroseptica,* brown rot of pineapple fruit by *E. ananas,* and crown rot of rhubarb by *E. rhapontici.*

Species of Corynebacterium Producing Plant Disease

It is somewhat surprising to find six species of plant pathogens allocated to the genus *Corynebacterium* of which the diphtheria bacillus is the type. This genus includes in general gram-positive rods which are usually slender and with some tendency to show irregularly stained segments or granules. All the plant pathogens are aerobic.

The plant pathogens of this genus may be divided into two groups. The first group contains six species which are reasonably typical of this genus as usually recognized. *Corynebacterium insidiosum* invades the fibrovascular system of alfalfa; *C. sepedonicum* produces the serious potato disease ring rot; *C. michiganense* causes a bacterial tomato canker; and *C. fascians* causes fasciated growths on the sweet pea.

Four other species of plant pathogens placed in this genus are unlike all others in that they possess polar flagella. This would seem to put them in the genera *Pseudomonas* or *Xanthomonas*, but they are definitely gram-positive and otherwise seem to fit the generic description. One of them, *C. hypertrophicans* apparently is the cause of the formation of witches brooms on a species of *Eugenia*. *C. flaccumfaciens* produces a wilt in beans.

Other Bacteria Pathogenic for Plants

Three other species of bacteria are known to produce plant disease, but are not satisfactorily allocated to genera even though their principal characteristics are well known. Burkholder in the sixth edition of Bergey has allocated them to the catch-all genus *Bacterium*. All of them are gram-negative and produce yellow-pigmented colonies. One is motile by means of a polar flagellum, and causes blight on the leaves of iris. It is named *Bacterium tardicrescens*. It seems probable that it should be placed in the genus *Xanthomonas*.

The organism causing the serious disease sweet corn wilt is named *Bacterium stewartii*. It likewise produces yellow colonies, but is non-motile. The same is true of *Bacterium albolineans*, causing white stripe and leaf scald of sugar cane. It would seem wise to have placed these species as non-motile species of *Xanthomonas*.

CHAPTER 38

The Hemophilic Group: The Genera *Hemophilus*, *Moraxella*

The hemophilic group of bacteria includes several genera, *Hemophilus, Moraxella, Noguchia,* and *Dialister*. All the species included are relatively slender forms which, at least when first isolated, require the presence of certain growth factors which are contained in blood and tissues. There are two of these accessory growth factors commonly known (x and v). The x factor is hemin, an iron-containing constituent of the hemoglobin of the blood, found also in some plant and animal tissues. It is thermostable, and it is not destroyed even at the temperature of boiling water. The v factor is present in blood and is produced by many tissues and cells, as by yeast. It is a phosphopyridine nucleotide, or coenzyme I. Some of the organisms belonging to this group require both factors consistently, some require x and not v, some v and not x, and some require one or both factors when first isolated, but after continued cultivation they may be grown in the absence of these factors. These x and v factors are probably significant in the metabolism of most kinds of cells; but most cells can manufacture them. The parasitic habit of the hemophilic bacteria and the easy accessibility of these compounds in the blood and tissues of the host has led to loss of ability of these organisms to synthesize these factors. If an organism, such as the influenza bacillus, requiring both factors is plated on a medium containing the x factor seeded also with an organism such as *Micrococcus aureus,* the colonies of the influenza organism will develop only immediately adjacent to the colonies of the coccus. The appearance as of moons about a planet has given to this the name of the satellite phenomenon. Evidently some substance diffuses from the coccus colony which meets the need of the influenza bacillus for the v factor.

The four genera included in the tribe *Hemophileae,* the hemophilic group, are *Hemophilus*, *Moraxella*, *Noguchia*, and *Dialister*. The first three contain species that are aerobic or facultative in culture, the *Dialister* species are obligate anaerobes. Species of *Hemophilus* and *Moraxella* are non-motile, those of *Noguchia*, motile. The two genera discussed are *Hemophilus* with cells usually single and *Moraxella* with cells in pairs.

The Genus Hemophilus

The cells of *Hemophilus* are minute slender rods, occasionally elongate and threadlike, non-motile, and gram-negative. The species are all strict parasites of man and animals. They grow best or only in the presence of the necessary growth accessory substances, or in blood serum or similar materials which contain them naturally. Seven species are recognized, of these the type species is *Hemophilus influenzae*.

The *Hemophilus influenzae* takes its name from the disease influenza. It was first isolated from cases of this disease and was supposed to be the cause. It was later found that influenza is caused by a virus; however, the *Hemophilus influenzae* is regarded as having pathological significance as a secondary invader. It has been shown to be the cause of a considerable percentage of the cases of infantile meningitis. It has been isolated from a great variety of situations, from sputum, the conjunctiva, pharynx, sinuses, cerebrospinal fluid, and from blood in cases diagnosed as influenza. When grown in artificial media it is found to require both x and v factors for growth. On blood agar the colonies are small, round, and transparent. It grows more luxuriantly on chocolate blood agar, made by heating blood to 90° C. for ten minutes, which seems to destroy some blood inhibitory principle. Some strains may produce acid from some carbohydrates, but the character is variable. The temperature optimum is blood heat. Six types of the influenza bacillus have been differentiated on the basis of the precipitation by immune antibodies of the type-specific capsular material.

Hemophilus suis is differentiated from *H. influenzae* on the basis of minor characters. It requires both growth factors. When associated with a specific virus in swine the disease swine influenza is produced. The virus attacks the mucous membranes of the respiratory tract, making possible the growth of the *H. suis*. It was emphasized by Shope, who discovered this relationship, that the two agents, the bacterium and the virus, must act together (an example of pathological synergism) to produce the disease swine influenza.

Hemophilus pertussis is the cause of the disease whooping cough. The short oval rods are 0.2–0.3 μ by 1.0 μ, usually single, occasionally in pairs or short chains. Bipolar staining is sometimes apparent. Capsules may be present. The organism does not grow on the usual laboratory media, but will develop on a medium containing a considerable proportion (15 per cent) of blood, particularly on the medium developed by Bordet and Gengou, who first described the organism. This medium consists of 1 per cent glycerol agar with macerated potato mixed with an equal volume of rabbit or human blood. The colonies on blood agar are smooth, elevated, and with a pearly luster. The colonies produce a zone of hemolysis about them. The organisms grow on the ciliated epithelium of the trachea. They occur in such large numbers that it has been inferred that much of the damage is due to mechanical interference with the movement and functioning of the cilia. There is not, therefore, the normal removal of the secretions.

The disease may be diagnosed by having the patient cough directly into a plate containing the Bordet-Gengou agar. If the organism is present the characteristic colonies will appear after incubation of the plate at blood heat for two or three days. The bacteria in their virulent form are in the smooth (S) stage. The rough (R) colonies develop upon culture. Vaccines are prepared by growing the smooth (S) bacilli of phase I of the organism and killing a suspension of the cells. Prophylactic injections have proved efficacious in preventing the disease in children, and vaccination is extensively employed. No antiserum has proved effective.

The *Hemophilus ducreyi* produces a venereal disease characterized by the development of soft chancres. This organism requires the x but not the v factor for growth. It resembles the other species of *Hemophilus*. It is slender, 0.5 μ by 1.5–2.0 μ. On blood agar a zone of hemolysis occurs about the colony. The disease is transmitted by direct contact.

The genus *Moraxella* differs in part from *Hemophilus* in that the rods are usually in pairs. It includes three species of bacteria that have been found in inflammation of the eye, two infecting the conjunctiva in man (producing conjunctivitis or pink-eye) and one causing keratitis (infection of the cornea) in cattle. The species important in man is *Moraxella lacunata*, often termed the Morax-Axenfeld bacillus, after the discoverers.

CHAPTER 39

Group of Spore-Bearing Rods: The Genera *Bacillus* and *Clostridium*

The pathogenic spore-producing bacteria are relatively few in number. They belong to the genera *Bacillus*, which includes the aerobic species and *Clostridium*, which includes the anaerobic forms.

The Genus Bacillus

Smith in his revision of the aerobic spore-producing bacteria includes in the genus *Bacillus* a total of thirty-three species. Until the appearance of this monograph the situation was somewhat chaotic, for great numbers of species of the genus had been described. For example, Smith was able to reduce to synonymy with *Bacillus subtilis* about twenty-five names of species that had been proposed in the literature.

The genus *Bacillus* includes rod-shaped bacteria that may be single or in chains. The spore-producing cells (sporangia) in most cases do not differ from the vegetative rods in size or shape. The enzyme catalase is present; hydrogen peroxide applied to the colonies will evolve gas. The organisms are aerobic, when motile; peritrichous flagella are present and the cells are usually gram-positive.

Bacillus subtilis, the type species, is a common inhabitant of soil. It is often called the hay bacillus because it was readily found by Cohn (who named the species) to appear in boiled infusion of hay. It is of interest therapeutically because from its cultures and those of several related species several kinds of antibiotics such as *subtilin* have been produced.

Another species, *B. cereus*, commonly distributed in soil is of special interest because of its close resemblance to the causal organism of the disease anthrax (*B. anthracis*). The resemblance is so close that some authors have held the latter to be a non-motile pathogenic variant of *B. cereus*.

Bacillus anthracis

This is the only important species of *Bacillus* pathogenic for man and higher animals. The organism is a *non-motile* rod 1.0–1.3 μ by 3–10 μ, with square or concave ends, usually in long chains. In morphology, as in cultural characters, it closely resembles *B. cereus*. Gelatin is liquefied. Colonies on agar are large, irregular, and with a curled structure composed of parallel chains of cells. Milk is coagulated and slowly peptonized. Acid is produced from some sugars, but no gas. Acetoin is present.

This organism is the cause of anthrax in cattle, sheep, swine, and man. The disease in cattle and sheep usually manifests itself as a bacteremia causing high fever and usually death of the animals infected within a few days. There are several foci of infection in the United States, in some of the western ranges and in certain southern states. The disease may be transmitted to man from handling hides of cattle that have died of the disease, or in sorting wool in which the spores of the organism may be present. When inoculated under the skin, as through a wound, it produces what is known as a malignant carbuncle which may heal spontaneously or may result in the general invasion of the blood stream and cause death from bacteremia. The inhalation of the organism may produce the so-called woolsorter's disease, a very acute and malignant type of pneumonia. During World War I there were some cases of anthrax in soldiers due to the use of shaving brushes made from hair (imported from the orient) that had not been properly sterilized. The fact that this organism produces highly resistant spores renders it difficult to eliminate. It has been known to persist for years in a pasture.

It is possible to attenuate a culture of *Bacillus anthracis,* by growing it under unfavorable conditions, to such an extent that it may be used effectively as a vaccine in the prevention of the disease. It is also possible to confer passive immunity by the use of antiserum from an animal that has been rendered highly immune. The disease is of great historical interest as the one in which the first adequate proofs of the causal relationships of an organism to a disease were worked out in the pioneer studies of Robert Koch.

Bacillus larvae

This organism is the cause of the destructive disease American foulbrood of the honey bee. The rods are 0.5–0.8μ by 2.5–5.0 μ, single or in chains, the sporangia swollen and spindle-shaped, and the spores central or near the end, ellipsoidal. The organism will grow in culture media to which carrot, yeast extract, or thiamin has been added. The organism attacks the larvae in the brood cells of the comb, killing them promptly. Adult bees are apparently not attacked. The nurse bees carry the organism about, so that comb and honey may be infective. The disease may spread rapidly through an apiary, wiping out most of the colonies. Drastic measures such as complete destruction of all infected hives and combs are usually required under bee inspection laws of the several states. Colonies of bees show distinct differences in their ability to resist or throw off the disease. This resistance is apparently inherited, and progress is being made in developing strains of bees which are able to combat the disease satisfactorily.

Bacillus popilliae

This organism is of interest because it is apparently the best agent at hand for coping in an adequate manner with certain insect pests. It produces the highly fatal milky disease of the larva or grub of the Japanese bettle and of certain related leaf beetles. The rods are relatively large, 0.9 μ by 5.2 μ. They are non-motile and gram-positive. The spores are cylindrical, 0.9 μ by 1.8 μ, contained in swollen, spindle-shaped sporangia. The sporangium contains also a refractive body about one-half the size of the spore, which stains in the same manner. The organism is cultivable on beef infusion agar to which has been added unheated egg yolk; small discrete colonies are produced. It grows best at about 30° C.

The Japanese beetle is a serious pest as a grub in lawns and pastures. The adult feeds upon leaves of many plants, and is particularly destructive to fruit such as the peach. The disease was first discovered in New Jersey. Quite probably it was a disease of some native grub which proved to be readily transmissible to the grub of the Japanese beetle (*Popillia japonica*). The exact origin is not known, although other strains of bacteria have been found to infect and cause disease in the grubs of various species of May beetles.

The Japanese beetles lay their eggs in short grass of lawns, pastures, and similar places. These hatch into grubs which feed largely upon roots. They are whitish and somewhat translucent. They may be infected with the milky disease by ingesting spores of the causal organism if these are present in the soil. When this occurs the bacteria multiply rapidly throughout the body, changing the normally clear blood to an opaque white fluid, whence the name, milky disease. The infection usually proves fatal, the bacterial cells sporulate, and are freed by the million into the soil by the disintegration of the grub.

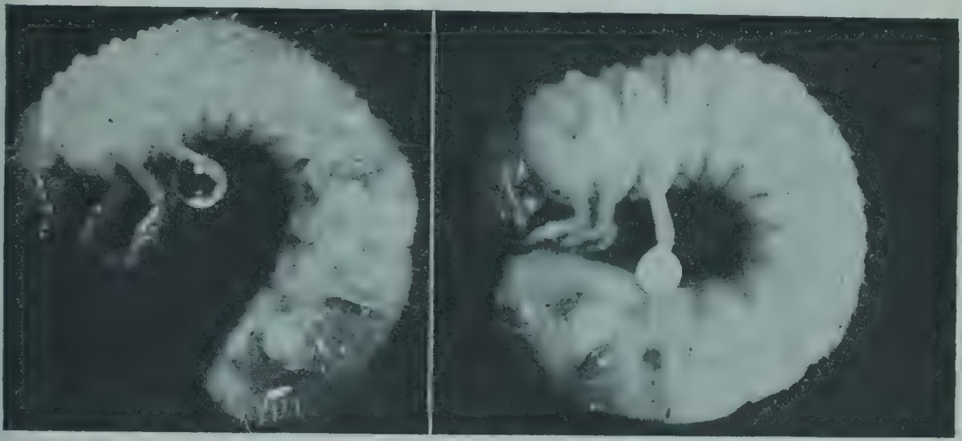

Fig. 39–1. Milky disease of larva of Japanese beetle. *Left.* Healthy larva. Note drop of clear liquid where leg has been cut, also transparency of the body. *Right.* Diseased larva. Note drop of milky liquid where leg has been cut, also the white opaque body. (Courtesy Agricultural Experiment Station, Geneva, New York.)

Some success in killing the grubs and controlling the beetles has been achieved by inoculating soil infested with the grubs with the spores of *B. popilliae*. The inoculum is prepared by gathering numbers of grubs infected with milky disease, grinding them with chalk, and mixing with talc to produce a powder having a definite concentration of spores. One method of application is to use about two pounds for an acre, usually distributed in spots about ten feet apart. About a teaspoonful of the dust is buried in each spot. The powder may also be broadcast. This is one of the few instances of successful control of a pest by means of a bacterial disease. The disease has been found to be transmissible to the larvae of many related beetles.

THE ANAEROBIC SPORE-PRODUCING BACTERIA

The Genus Clostridium

This group includes a considerable number of species belonging to the genus *Clostridium*. Many of them are not uncommon in the soil, most of them are non-pathogenic. Spray (1942) in the sixth edition of Bergey's *Manual* recognized sixty-one species, a large number were made synonyms, and a hundred ninety-four additional names of species are listed which are too inadequately described to be either identified or reduced to synonymy. Of these about twenty are capable of producing diseases, or have been isolated from pathological conditions.[1] Among these are *Clostridium tetani*, producing tetanus or lockjaw; *C. botulinum*, causing a type of food poisoning called botulism; *C. aedematis*, the cause of malignant edema; *C. chauvoei*, the cause of black leg in cattle; and *C. perfringens*, which has been found in connection with a few cases of disease in man as a probable cause of gaseous edema. *C. tetani* and *C. botulinum*, of importance in the causation of tetanus and botulism respectively in man, will be discussed.

The organisms of this group are placed together because of their failure to develop in the presence of an abundant supply of oxygen. All produce spores, and most of them develop gas from carbohydrates and some from proteins.

Clostridium tetani

Clostridium tetani is the cause of tetanus or lockjaw in man and certain of the domestic animals, particularly the horse. It was discovered in 1889 by Nicolaier, who found it in pus from animals that had died as a result of the subcutaneous inoculation of a suspension of rich garden soil. He succeeded in growing the organism in artificial media, but could not get it in pure culture. Kitasato in 1889 secured pure cultures by use of anaerobic techniques and proved experimentally that this organism was the specific cause of the disease tetanus. Together with Veyl in 1890 he demonstrated the production of a specific toxin in culture media. This organism is not uncommon in the alimentary tract of herbivorous animals, particularly the horse, is frequent in manured soil, and is practically always present in street dust. It is not certainly known whether it can multiply in nature outside of the body, but this seems to be probable. It is perhaps to be regarded as a normal saprophyte that is capable of producing disease only under exceptional conditions.

Morphology and Culture. *Clostridium tetani* is a long, slender, motile rod 0.5 μ by 2.0–5.0 μ. It usually occurs singly, but occasionally it appears in short chains. Capsules are not formed. Spores are produced abundantly under almost all conditions. These spores are several times the diameter of the rods within which they are borne. The spores occur at the ends of the rods, giving a characteristic drumstick appearance. The organism stains readily and is gram-positive. The fact that this organism is an obligate anaerobe renders it somewhat difficult to cultivate. It was first isolated by producing tetanus in experimental animals, inoculating a broth culture from the pus of the wound and heating to 80° C. for one-half hour. The spores of the organisms will resist this temperature, while other bacteria present will be destroyed. Transfer from this broth into nutrient agar or gelatin kept under anaerobic conditions will usually show development of the tetanus bacillus. The organism grows readily upon most of the culture media. A stab culture in gelatin shows an arborescent, radiating growth along the line of stab. Gelatin and blood serum are liquefied and milk is coagulated by the production of acid.

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[1] The species of pathologic interest are *Clostridium fallax*, *C. septicum*, *C. feseri*, *C. novyi*, *C. botulinum*, *C. aerofoetidum*, *C. sporogenes*, *C. parabotulinum*, *C. perfringens*, *C. sphenoides*, *C. innominatum*, *C. paraputrificum*, *C. cochlearium*, *C. capitovale*, *C. tetanoides*, *C. tetani*, *C. chromogenes*, *C. tetanomorphum*, *C. histolyticum*, *C. tertium*

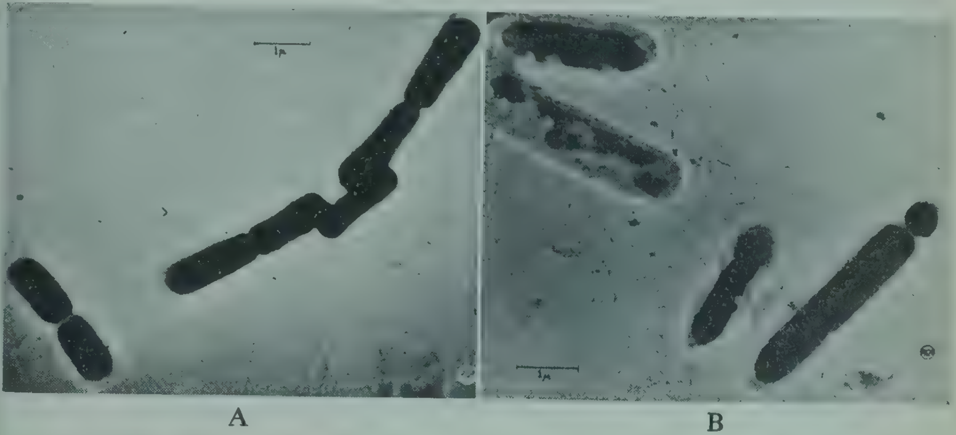

Fig. 39–2. *a. Clostridium botulinum.* Electronograph. Note the faint peritrichous flagella. The organism shows no spores and seems to be homogeneous. *b. Clostridium tetani.* Electronograph. Some cells are intact, others have undergone some degree of autolysis. Note the numerous peritrichous flagella. One intact cell shows unequal cell division and sharp differentiation of the cytoplasm and cell wall. Other cells show varying degrees of degeneration and cytolysis. (Courtesy of H. Mudd and T. F. Anderson and the *Journal of the American Medical Association.*)

The optimum growth temperature is blood heat, although the organism grows well at room temperature. It develops only when oxygen is almost wholly excluded from the medium. It will also grow under aerobic conditions when mixed with aerobic bacteria. The spores are quite resistant to drying and to other unfavorable conditions. In some cases the resistance to heat is so great that exposure to live steam for over an hour is not sufficient to destroy them certainly.

Disease Production. The disease is most common in man and the horse. It is ordinarily the result of wound infection. The organism gains entrance to a wound, particularly one which is relatively deep and heals or closes superficially, and there produces its characteristic toxin. This is taken up by the motor nerve endings, passes along the nerve trunks to the central nervous system; probably in part it also passes to the blood stream and is carried by this means likewise to certain portions of the central nervous system. The nerve cells are injured, and the muscles of the body, particularly those of the part affected, contract and become rigid. They are said to go into a state of *tetany*. The bacillus rarely gains entrance to the circulating blood and does not develop in the tissues to any great distance from the point of inoculation. The growth of the organism, the development of the toxin, and the absorption of this toxin by the nerve cells is so slow that the period of incubation in man averages about nine or ten days. In the horse it may be somewhat longer. The disease is not contagious. The only common means of infection, as has been stated, is through a dirty wound. The puncture from a rusty nail, for example, is infective not because of the rust, but because of the dirt containing spores of the tetanus bacillus which is likely to be present upon its surface. These spores are carried deep into the wound by the rough surface of the nail and are left there upon its withdrawal. Bright or clean instruments are not as likely to transmit the disease because there is greater opportunity for the organism to be brushed aside by the skin and because they do not ordinarily have the specific organism upon their surfaces. An important point to be noted is the fact that when the spores of the tetanus bacillus are obtained entirely free from their characteristic toxin by repeated washing, pure cultures may be injected subcutaneously into animals without producing the disease. The organism finds its most favorable conditions for development when associated with dirt in wounds, and particularly with some of the pus-producing bacteria. Apparently this organism grows largely in injured or necrotic tissues. It has little power to penetrate healthy uninjured cells.

Immunity. Tetanus is a toxemia. It is possible, therefore, to produce antitoxins for the disease. The toxins are developed readily in culture flasks of broth kept under anaerobic conditions. After incubating for a week or more, the broth is filtered to remove the bacteria. The toxin may be concentrated from the broth by means of precipitation by ammonium sulfate. By such methods toxins may be secured so potent that 0.00000025 gram is sufficient to kill a white mouse. It has been shown that the toxin has two poisonous constituents, one termed *tetanolysin* that is capable of destroying red blood corpuscles, and the other *tetanospasmin* which has a particular affinity for the nerve cells and which is the immediate cause of the characteristic symptoms of tetanus. The antitoxin is prepared by the injection of increasing doses of the toxin into a healthy horse. After a time the animal may develop a high grade of immunity. Blood may then be drawn, the serum removed and utilized as a means of preventing the disease in man. It is now customary for physicians to inject antitoxin as a preventive whenever there is a wound. Inasmuch as the symptoms of tetanus become apparent only after a considerable proportion of the damage has been done, it is evident that the injection of antitoxin into an individual already showing these symptoms cannot undo the damage already done. In other words, while tetanus antitoxin is an excellent prophylactic and its injection will practically insure the prevention of tetanus, its usefulness as a curative agent is not so pronounced.

Active immunization against tetanus is extensively practiced by use of toxoid injections. The toxoid is prepared from toxin in various methods, as by the addition of formaldehyde, which destroys most of the toxic effect of the toxin. The toxoid acts as an antigen when injected, causing the development of an active immunity. Army experience during World War II seems amply to warrant its use.

Clostridium botulinum

Synonym. *Bacillus botulinus.*

This organism is one of the frequent causes of meat and food poisoning in man. The disease is generally termed botulism (from the Latin *botulus,* sausage). The organism was first isolated by Van Ermengen in 1896 from a sausage which he believed to be the cause of an outbreak of food poisoning. Poisoning caused by this organism should not be confused with those caused by *Staphylococci* or *Salmonellae*.

Distribution. Outbreaks of poisoning of the type of botulism have been repeatedly reported from Europe, particularly as the result of eating meat foods and sausages. In the United States practically all the cases reported have been from the eating of imperfectly preserved canned foods. A considerable variety of these have been found to be at fault. Cases of poisoning from canned asparagus, canned beans, and ripe olives have been reported. Most of the cases have been from the Pacific coast. Botulism sometimes occurs in birds, particularly in wild ducks that have eaten decaying insects in the alkaline marshes of the western parts of the United States.

Morphology. *Clostridium botulinum* is a relatively large bacillus, usually single, occasionally in pairs or chains. It is 0.9–1.2 μ by 4–6 μ. It is actively motile by means of from four to eight peritrichous flagella. Oval or elliptical spores are produced, one near the end of each cell. The organism stains readily and is gram-positive.

Culture. Care must be taken to exclude oxygen in the growth of *Clostridium botulinum*. It grows fairly readily in ordinary culture media.

Physiology. The organism is an obligate anaerobe. Some strains grow best at room temperatures, others at body temperatures. Apparently there are several distinct varieties of this organism which show minor differences in physiological and immunological reactions. Gas is generally produced from dextrose, but not from sucrose or lactose. The spores are quite resistant to heat, and their presence in food which has been canned is a major health hazard.

Pathogenesis. When growing under favorable conditions, *Clostridium botulinum* produces a highly potent toxin. Commonly this is accompanied by the formation of malodorous substances, particularly butyric acid. Usually food in which this organism has been growing can be detected by having an "off" odor. Such foods must be carefully avoided, as even a small portion of such food, sometimes a mere taste, has proved fatal.

The toxin produced by growing the organism in culture media is quickly fatal to laboratory animals when fed or injected. It is also frequently fatal to man. In man the symptoms include lack of coordination of the eyes, that is, double vision, and difficulty in control of the muscles governing swallowing.

Until recently it has been believed that the organism itself (free from toxin) when it has been introduced into the animal body is non-pathogenic, but more recent work seems to show that the organism when injected or fed to certain animals is capable of producing serious or even fatal results.

Limber-neck of chickens may be caused by feeding food containing the organism or its toxin. The birds are partially paralyzed, resting the tip of the bill upon the ground, and showing lack of ability to hold the head erect.

Immunity. *Clostridium botulinum* produces one of the most powerful toxins that has been described; 0.00005 gram has been found to produce death in three to four days when injected subcutaneously into a guinea pig, and ten times this amount will destroy a rabbit. It is to be sharply differentiated from the toxins of the tetanus and diphtheria bacilli in that it can produce characteristic symptoms when introduced by way of the alimentary tract, whereas the toxins of both the tetanus and diphtheria bacilli are rendered innocuous by the digestive juices. This toxin has the various characteristics which have been listed by Ehrlich as determining a true toxin. It is easily destroyed by exposure to light and air, and heating to a temperature of 80° C. renders it inactive. It is evident therefore that the heat of cooking is sufficient to destroy the toxin.

Inasmuch as this organism produces a toxin, it is possible that the corresponding antitoxin could be used in treating the disease. In animals it has been used effectively in curing the disease; it has been shown to be of value in man. There appear to be at least two distinct strains of *Clostridium botulinum* whose toxins are specific; it is necessary therefore to utilize the antitoxin homologous to the organism causing the disease.

Since this organism has been found to infect man most frequently from canned foods, it is of interest to note that the disease has resulted both from commercially canned and home-canned foods. It is highly important therefore that canned foods be adequately sterilized, and care should be used not to consume foods which are "off" in odor.

CHAPTER 40

Acid-Fast or Tuberculosis Group: The Genus *Mycobacterium*

The principal organisms belonging to the acid-fast group of bacteria are *Mycobacterium tuberculosis*, the cause of tuberculosis in man and animals, *M. avium* of tuberculosis of birds, *M. paratuberculosis* of Johne's disease or paratuberculosis in cattle, several species pathogenic for amphibians and reptiles, *M. leprae*, the specific cause of leprosy, and some non-pathogenic organisms found not uncommonly in soil, feces, butter, milk, etc. The term *acid-fast* (or *acid-proof*) is used to indicate that these organisms are somewhat difficult to stain, but when once stained with the aniline dyes such as fuchsin, they retain the stain with great tenacity, even when treated with relatively strong solution of inorganic acids, such as sulfuric, which would decolorize other kinds of bacteria completely. The chemical nature of the cell substances which are responsible for the acid-fastness of the members of the genus *Mycobacterium* has been studied by many investigators. It appears to be due to the presence in the cells of mycolic acid, a long chain hydroxy fatty acid. Apparently the acid-fastness is dependent in part upon the free carboxyl group being present, for the esters of the compound are not acid-fast. Mycolic acid has been prepared from *Mycobacterium tuberculosis*, and similar acid-fast compounds from related acid-fast bacteria. In the cell the mycolic acid is apparently rather firmly bound to other cell constituents. The cells of all mycobacteria are slender, non-motile rods, never producing capsules or spores, and are gram-positive.

Mycobacterium tuberculosis

Synonyms. *Bacillus tuberculosis, Bacterium tuberculosis.*

This organism is the cause of tuberculosis in man, and other mammals. The disease in its various manifestations has been known since ancient times. The several different types of tuberculosis, however, differ so much clinically that their relationship was not even suspected until comparatively modern times. The possibility of transferring the disease from one animal to another was demonstrated by Villemin in 1865, but he did not discover the causal organism. The specific etiologic agent remained in doubt until the publication of the work of Robert Koch in 1884, in which he described the tubercle bacillus. Koch succeeded not only in demonstrating the presence of this organism in the various lesions of the disease, but also in cultivating it upon artificial culture media. The latter is especially noteworthy, inasmuch as this organism is not easily grown. The presence of acid-fast bacteria in tuberculosis both in man and other animals, and the close resemblance of the organisms, led to the assumption that all tuberculosis was caused by the same bacterium. In 1896 Dr. Theobald Smith noted that it was possible to differentiate the bacteria causing tuberculosis in cattle from those usually present in the disease as it occurs in man. Even earlier it had been shown that the organism producing avian tuberculosis is different. Koch in 1901 read a paper before the International Tuberculosis Congress in which he asserted that the bovine and human tubercle bacilli are distinct and that probability of transmission from the bovine to man is so small as to be negligible. This ran counter to the general opinion among bacteriologists and sanitarians, and led to intensive studies. These resulted in the demonstration that there are three distinct types of tubercle bacilli, the *human,* the *bovine,* and the *avian*. The names *Mycobacterium tuberculosis* variety *hominis* and *M. tuberculosis* var. *bovis* recognize the first two as varieties of the species. The avian organism is usually recognized as a distinct species, *Mycobacterium avium.*

Tuberculosis is one of the most important of the bacterial diseases affecting men; formerly it caused more deaths than any other single disease. In the early part of the nineteenth century about 20 per cent of all deaths in western Europe and the eastern United States were due to tuberculosis. In some cities death rates (number of deaths per year) were as high as 500 per 100,000 population. Since that time there has been a steady decline to less than 50 per 100,000 in the United States, and even lower in some countries such as Denmark. There are areas of the world, however, in which the disease is still the most common cause of death. Tuberculosis is also widely distributed in cattle. In the United States it was once quite common in both dairy and beef herds, but systematic diagnosis and removal of infected animals has brought the disease reasonably well under control. Formerly in some localities from 10 to 25 per cent or even more of the milk cows have been found to be affected with disease. In some European countries the percentage is much higher. Swine also contract tuberculosis when fed milk from tuberculous animals or when allowed to run in the feed lot together with tuberculous cattle.

Morphology and Culture. *Mycobacterium tuberculosis* is a slender rod with rounded ends. It may be straight or somewhat bent. It measures 0.2–0.5 μ by 1.5–3.5 μ. Occasionally much longer filaments are found. It is usually single, almost never occurring in chains. The protoplasm often takes the stain irregularly, giving the cell a beaded appearance. It does not produce spores or capsules and is non-motile. Involution forms, such as branched and club-shaped rods, are sometimes observed. The organism stains with difficulty with the ordinary aniline dyes. It is generally necessary to heat the dye or to allow it to remain for a long time in contact with the organism before it stains sufficiently for observation. When once stained, however, the application of acids will not decolorize the cells. This character is of value in the diagnosis of the disease, as it renders recognition of the organism in sputum or tissues possible.

M. tuberculosis is isolated from the lesions of the disease with a considerable degree of difficulty, inasmuch as it does not grow readily upon most of the culture media, at least at first. Whenever it occurs mixed with another organism, animal inoculation must generally be resorted to in order to secure a pure culture, the organism then being isolated from the characteristic lesions. Pure cultures are secured by rubbing bits of infected tissue containing the nodules or tubercles over the surface of coagulated blood serum and incubating at blood heat. The organism does not usually show any growth for a period of a week or more. The colonies appear at first as tiny grains, barely visible to the naked eye. These gradually enlarge and may become confluent.

It also has been found possible to treat mixtures of tubercle bacillus and other organisms, as in sputum, with strong alkalies which will destroy the other bacteria, but leave the tubercle bacillus alive. After cultivation for a time upon protein medium, transfers may be made to glycerol agar. Upon this medium it is usually possible to differentiate the bovine and human tubercle bacilli from each other. The bovine bacillus develops characteristically as discrete colonies which rarely fuse to form a continuous layer over the surface. The human bacillus, on the contrary, grows much more luxuriantly; the colonies fuse, and the growth becomes wrinkled. The bacillus is quite easily cultivated on coagulated egg though growth is slow. On glycerol broth, the growth appears as a more or less heavy pellicle upon the surface. It is easily broken to pieces and settles to the bottom when shaken.

The tubercle bacillus and acid-fast bacteria, in general, give evidence of possessing surface membrane differing markedly from those of most other groups of bacteria. They belong to that type of particle which in suspension in water is termed hydrophobic.[1] Their surfaces are not readily wetted by water—the cells behave as though they were somewhat waxy or greasy. In culture, as in the body, the cells tend to stick together rather than disperse readily in water or in the usual liquid culture media; they form clumps or pellicles. This characteristic makes it difficult to prepare reasonably homogeneous suspensions that would be useful for many purposes in the laboratory. Bacteria of most other groups have surfaces which are easily wetted, indeed the capsular material tends to absorb water. Such are said to be hydrophilic (water-loving). The tubercle bacillus may be dispersed in the medium by the addition of a suitable *wetting agent,* or detergent. Most of these, however, are toxic to the tubercle bacillus, inhibiting growth. There have been found (Dubos) so-called surface active substances or wetting agents which still cause deflocculation when used in dilutions so great as no longer to be inhibitory. These belong to a group of non-ionic wetting substances, i.e., they are not soaps. One of them, Tween 80, is apparently adsorbed to the surface of the cell by its long aliphatic chain, and the ethylene oxide chains make the new surface hydrophilic. In the presence of albumin in a liquid medium a relatively luxuriant growth of dispersed cells may be secured. Such dispersed cells are much more susceptible to various antibacterial substances. For example, in the usual media tubercle bacilli will grow in the presence of one hundred micrograms of penicillin per milliliter, but in the presence of a suitable wetting agent they are inhibited by five micrograms.

The tubercle bacillus is aerobic. It has an optimum temperature of about 37° C. and a limited growth temperature range. After long cultivation upon artificial media, growth may occur at somewhat lower temperatures. The thermal death point for the organism is 60° C. for twenty minutes. Considerable confusion exists in the literature relative to this thermal death point because of difficulties in the method of determination. Its accurate determination is of particular interest in the pasteurization of milk. The work of members of the Hygienic Laboratory of the United States Public Health Service seems to indicate that pasteurization of milk at the temperature noted above will certainly destroy the tubercle bacilli present, provided the vessel in which pasteurization is carried out is closed so that no scum can form on the surface of the milk. When the tubercle bacillus is dried in sputum, it is quite resistant to desiccation and to the sun's rays.

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[1] Literally water repelling.

The principal physiologic character which has been suggested as useful in the differentiation of human and bovine tubercle bacilli is that of the reaction produced in glycerol broth. This was carefully worked out by Theobald Smith, who came to the conclusion that in glycerol bouillon the human bacillus brings about a permanent acid reaction, whereas the bovine bacillus causes the reaction to become alkaline in the course of time.

Disease Production. Tuberculosis is a disease to be classed among chronic infections. V. A. Moore states, "It does not destroy life by acute toxemia, but by a chronic and long-continued systemic poisoning and by the morbid changes brought about through the localization of these lesions in the organs necessary to life."

The disease gets its name from the production of the characteristic nodules or tubercles in the tissues affected. The organism sets up a local inflammation in any tissues to which it gains entrance and brings about at that point a gradual formation of several more or less concentric layers of cells. The structure of the nodule is practically the same no matter what the tissue affected.

Tuberculosis is easily transmitted to laboratory animals. In general, the bacillus of bovine origin is more virulent than that from man. For example, a subcutaneous injection of bovine bacilli into guinea pigs generally causes death in less than five weeks, while those inoculated with the human bacillus usually live a longer period. Intraperitoneal injections of bovine bacilli are commonly fatal in from one to three weeks, while the human bacillus generally produces death in from ten days to five or six weeks. Even more marked differences are to be noted in the rabbit. Bovine bacilli injected intravenously into this animal generally produce death within three weeks, while with human bacilli, rabbits commonly live for several weeks and frequently recover completely. It is also found that human bacilli rarely or never produce tuberculosis when injected into cattle, even into calves. This character is the most important and probably the most reliable of the methods used in differentiating the two organisms. It has been found, however, that both human and the bovine tubercle bacilli can produce tuberculosis when fed to the monkey.

Practically any part of the body may be affected in tuberculosis. The lymphatic system is usually involved. As the nodules enlarge they often coalesce and the interior becomes cheeselike in consistency (undergoes caseation). In some cases the nodules may become surrounded by a capsule of fibrous tissue, quite efficiently preventing the escape and spread of the organism. In other cases the nodules may ultimately become calcified. These calcareous nodules are not infrequently found in healed tuberculous areas. Within the body the bacteria are probably most frequently spread by the lymphatics, sometimes also by the blood stream.

In man the tubercle bacillus most frequently attacks the lungs (consumption). Tuberculous infections of the glands of the neck (scrofula), of the bones and joints, of the alimentary tract and abdominal organs, such as the liver, spleen, and kidneys, and even of the brain covering (tubercular meningitis), or of the skin (lupus) are also caused. In cattle the nodules appear most frequently upon the membranes lining the peritoneal cavity and covering the intestines. They are not infrequent in the lungs and accompanying lymph glands. Of particular importance is the occasional development of tuberculosis in the udder of the cow. The percentage of tuberculous udders among tuberculous cows is not certainly known. Some authors believe it to be less than 1 per cent, others estimate it as high as 5 per cent. Swine are most commonly infected in the lymph glands of the neck (scrofula) and in the abdominal organs.

Immunity. The tubercle bacillus does not form true toxins, hence antitoxins cannot be produced. Poisonous substances, possibly of the nature of endotoxins, are formed. They are probably released in large part only upon the dissolution of the bacterial cell. There is commonly a considerable degree of natural immunity to tuberculosis, for a large percentage of individuals infected with this organism recover from the disease. This is true both in man and animals. It has not been shown, however, that those who have recovered in this manner are resistant to new infection. To what immunity in this disease is due is not certainly known. Some use has been made of tuberculin injected subcutaneously in minute quantities into those affected with tuberculosis in an effort to stimulate the production of antibodies and the development of immunity.

There may be some positive correlation between morphology of *M. tuberculosis* and the strain virulence. Dubos (1949) has concluded that in general a difference is manifest in suitable culture media between virulent and avirulent strains. The virulent forms tends to adhere to each other with cells parallel, forming ropes or strands which may be relatively long. The avirulent forms tend to show no definite orientation of cells, they are randomly arranged.

The most extensively used vaccine against tuberculosis is that usually designated as BCG (bacille Calmette-Guérin) prepared from a culture of *M. tuberculosis* var. *bovis* which had been subcultured more than two hundred times during a period of thirteen years. The intent, of course, was gradually to decrease the virulence of the organism through long cultivation on artificial media. The procedure yielded a strain, probably a mutant, which is not virulent for man or cattle. A vaccine consisting of a suspension of these organisms has been used extensively in France and elsewhere in Europe in immunization of children and of cattle. Apparently the vaccine is safe, at least the injected avirulent organisms do not produce tuberculosis. The usefulness of the vaccine even after several decades of study is not too well proved, although there are many who advocate its employment.

Diagnosis. Tuberculosis is diagnosed usually by one of three methods, by staining, by animal inoculation, and by the tuberculin test.

Diagnosis by Staining. Use may be made of the acid-fast characters of the tubercle bacillus in recognizing it in stained mounts. Sputum, for example, from an individual suspected of having tuberculosis may be spread upon a glass slide, dried, and fixed, then stained with hot carbol fuchsin for several minutes. This stains both the tubercle bacilli and all other organisms and cells present in the sputum. The mount is treated with acid alcohol or solutions of sulfuric or nitric acids. This removes the color from all organisms not acid-fast. After washing in water, the counter stain may be made by means of methylene blue. Upon examination under the microscope, the tubercle bacilli are found to be bright red in color, all other bacteria and cells being stained blue. This staining method cannot be used in the recognition of tubercle bacilli under all conditions, inasmuch as there are many other acid-fast bacteria that are not uncommonly found in milk, feces, and soil, etc. These rarely if ever are present in the sputum. The recognition of acid-fast bacteria in this material is almost certainly diagnostic of tuberculosis. Numerous methods of concentrating the tubercle bacilli to increase the reliability of the test have been developed.

A useful method of recognizing tubercle bacilli is to stain sputum or other materials with the fluorescent dye auramine (solution in 3 per cent phenol in water), heat, then decolorize with acid alcohol, and examine by means of ultraviolet light. This light causes the stained bacteria to give off a yellow light (to fluoresce) and to stand out in contrast to the unstained and non-fluorescent background.

Diagnosis by Animal Inoculation. The injection of material containing tubercle bacilli into the guinea pig will result in the development of tuberculosis in this animal within a few weeks. The organism may be isolated from the characteristic nodules of the diseased animal. The most certain method of detecting the presence of tubercle bacilli in milk is by means of animal inoculation.

Diagnosis by Tuberculin. Tuberculin is the name applied to a suspension of dead tubercle bacilli or a solution of their products. It has been prepared in many different ways, some types of tuberculin being used only for diagnostic purposes, others, as has been noted above, in the treatment of the disease. That most commonly used is Koch's Old Tuberculin. This is prepared by inoculating flat-bottomed flasks containing 4 per cent glycerol broth with a culture of *M. tuberculosis*. Care is used in making the transfer to make sure that the organism floats on the surface of the medium. It spreads out in growth until a heavy film is developed, which finally becomes much wrinkled. The flask is incubated until the maximum amount of growth has developed, usually for about eight weeks. The contents of the flask are then sterilized by heat and evaporated down to about one-tenth of the original bulk. The mixture constitutes tuberculin. In some cases this is passed through a porcelain filter to remove the bacterial cells. The tuberculin then contains only the soluble products of the bacterial growth. Other types of tuberculin are prepared by drying, grinding, and extracting the tubercle bacilli with water, and still others by precipitation with alcohol and re-solution of the precipitate in water.

Tuberculin is used in several ways in the diagnosis of the disease. The test as commonly applied to cattle is to inject the equivalent of 0.25 cubic centimeters of Koch's Old Tuberculin subcutaneously.

The temperature of the animal is taken at a sufficient number of intervals before the injection to ascertain the normal for the individual. Beginning six or eight hours after the injection, the temperature is again taken every two hours during the remainder of twenty-four hours. An animal having tuberculosis will show a fever reaction which reaches its maximum intensity usually in ten to eighteen hours after injection and should show at least 1° F. rise in temperature.

It was noted above that all the tissues of the body of an individual having tuberculosis are sensitive to the tuberculin. This fact has led to the development of several tuberculin tests for use in man. The cutaneous tuberculin test of Von Pirquet has been much used with young children. The skin on the under side of the forearm is washed with ether and a drop of tuberculin is applied. The skin is slightly scarified, and the tuberculin is rubbed in by means of a bit of cotton. An individual with tuberculosis will develop a papule somewhat resembling that produced in smallpox vaccination.

Tuberculosis in cattle and in fowls may be diagnosed by means of the intracutaneous or intradermal tuberculin test. In this method the tuberculin is injected into the skin (not under). Its resorption is slower and, if the animal or bird is infected, a marked swelling develops. The skin at one side of the tail is usually chosen for the test in cattle, and the wattles in the fowl.

Tuberculosis is generally classified into two types, open and closed. In open tuberculosis the bacteria are constantly leaving the body, because the lesions lie near some of the channels of exit. In closed tuberculosis the bacteria do not leave the body, because the lesions do not open into any channel communicating with the surface.

Paths by Which Tubercle Bacilli Leave the Body. Tubercle bacilli are commonly present in the mouth and sputum of a person affected with pulmonary tuberculosis. This is probably the commonest channel by which the organism leaves the body in this disease. Coughing has been shown to throw off so-called infectious droplets, that is, particles of saliva or sputum containing tubercle bacilli. These usually settle quickly, but may remain in suspension for a sufficient period to allow of their inhalation by others in the immediate vicinity of the patient. The tubercle bacilli within the sputum can retain their vitality for a considerable length of time even when the sputum is dried, ground, and blown about as dust. More important that either infectious droplets or dried sputum in the spread of the disease is the use of infective drinking vessels or utensils for human food. The use of the public drinking cup has been prohibited in several of the states, owing to the danger of the transmission of the disease.

Tubercle bacilli are also swallowed and appear in the feces. In cattle tubercle bacilli from the lungs are usually swallowed, therefore both pulmonary and intestinal tuberculosis in these animals causes the appearance in the feces of a considerable number of the organisms. Probably most of the organisms leave the body of the cow in this manner. Tuberculosis in swine is quite regularly produced if they are allowed to run with tubercular cattle. Tubercle bacilli in milk from tuberculous animals would appear to be the commonest method of transmitting the disease from cattle to man. It has already been noted that tuberculosis of the udder occurs in a small percentage of the cows having tuberculosis. Some authorities believe that the tubercle bacilli may gain entrance to the milk even though the udder is not tuberculous. This must be regarded at the present time as not proved. Probably the commonest source of tubercle bacilli in milk is not from a tuberculous udder, but from bacilli excreted in the feces. When the bacteria are leaving the body in this manner, they must occur commonly upon the surface of the body and upon the udder so that they easily gain admittance to the milk during the process of milking.

Portals of Entry in Tuberculosis. There seems to be little question but that tuberculosis in man is frequently acquired by inhalation of the tubercle bacilli. The second most common method of infection probably is by ingestion. It was formerly believed that pulmonary tuberculosis must be due to inhalation of the tubercle bacilli, and tuberculosis of the abdominal organs, particularly the intestines, due to ingestion. It has been shown, however, that when tubercle bacilli are fed in considerable numbers to a young animal, they may be demonstrated within a few hours in the thoracic duct. In other words, the organism seems to be able to penetrate the intestinal wall of a young animal under favorable conditions, first gaining entrance to the lymph channels and then to the blood stream, and so infecting the lungs. Diseased tonsils also offer opportunity for infection of the neighboring lymph glands and the consequent production of scrofula. Occasionally tuberculosis may follow direct inoculation of the skin. This has sometimes been reported in butchers and veterinarians.

Intertransmissibility of Bovine and Human Tuberculosis. Is bovine tuberculosis transmissible to man? The variety *M. hominis* rarely causes disease when injected into cattle. Should it be true that bovine tubercle bacilli cannot be transmitted to man, many of our sanitary regulations concerning the use of milk and meat containing bovine tubercle bacilli might be relaxed. The answer seems to be definite. The var. *bovis* can produce disease in man. The accompanying table,

Diagnosis	*Adults 16 years and over*		*Children 5 to 16 years*		*Children under 5 years*	
	Human	*Bovine*	*Human*	*Bovine*	*Human*	*Bovine*
Tuberculosis of lungs	644	1(?)	11		23	1
Lymph glands and neck scrofula	27	1	36	21	15	21
Abdominal tuberculosis	14	4	8	7	9	13
Generalized tuberculosis of alimentary tract	6	1	2	3	13	12
Generalized tuberculosis	29		4	1	43	5
Tuberculosis of bones and joints	27	1	38	3	26	
Other types	30	2	18	1	86	13
Total	777	10	117	36	215	65

adapted from a paper by Parke and Krumwiede, summarizes the results from a large series of cases that have been carefully investigated. It is evident that the disease caused by the bovine variety is common enough in children under sixteen years of age to justify all reasonable precautions against the consumption of tuberculous meat and milk The probability of adults becoming infected with bovine tuberculosis seems to be rather remote.

Mycobacterium avium

This organism resembles the tubercle bacilli just described, bu has somewhat higher optimum temperature (40° C.). It may be differentiated culturally by the absence of tendency to form a pellicle o liquid media and by the moister, slimier growth on solid media. It i the common cause of tuberculosis in the domestic fowl. The pheasan among wild birds is susceptible, but domestic ducks, geese an pigeons are relatively resistant. The disease may be transmitted t swine, usually causing tuberculosis of the lymph nodes of the nec and abdominal cavity. The disease is sometimes sufficiently widespread in hogs to lead to heavy condemnation of meat in packin plants. Rabbits are highly susceptible, guinea pigs refractory, an goats, cats, and dogs quite resistant. The disease probably is not transmissible to man.

Mycobacterium paratuberculosis

This organism was observed in cases of chronic dysentery in cattle in Germany by Johne and Frothingham and is frequently known as Johne's disease. The organism was recognized as distinct from the tubercle bacillus by Bang (1906), but, because of the resemblance of the organisms and lesions, the disease was termed paratuberculosis.

The organism is an acid-fast, rather short, thick rod, 0.5 μ by 1–2 μ. It resembles the tubercle bacillus.

The organism was cultivated only after it was found that it required some growth substance which could be supplied only by addition of killed acid-fast bacteria or by their extracts such as tuberculin. Extracts of non-pathogenic acid-fast bacteria such as the timothy bacillus (*Mycobacterium phlei*), may be used. The active growth factor has been identified as phthiocol, a substance which also has vitamin-K activity in animals. Growth on special media such as glyerol agar or glyerol egg medium with the addition of the needed stimulant is very slow; visible colonies may require four to six weeks for development.

The *M. paratuberculosis* causes chronic diarrhea in cattle and sheep. Laboratory animals as guinea pigs, rabbits, mice, and rats are susceptible. There is no evidence of transmission to man.

The disease is diagnosed usually by an intravenous injection of Johnin, a diagnostic agent prepared from cultures of the *M. paratuberculosis* in the same manner as tuberculin is prepared from culture of *M. tuberculosis*. A positive reaction is evidenced by restlessness of the animal, fever, shivering, and diarrhea, fever usually reaching its maximum from four to eight hours after injection. Interestingly, a tuberculin made from the avian tubercle bacillus may also be used in diagnosis.

Mycobacterium leprae

Mycobacterium leprae is the probable cause of leprosy in man. The organism was discovered by Hansen in 1874 and described at greater length by Neisser and Hansen in 1880. The disease is a common one over a large portion of Asia and northern Europe (particularly the Scandinavian countries), in certain of the Pacific islands, and is occasionally found in the United States. Most of the cases in the United States are imported from Europe.

Morphology and Culture. *Mycobacterium leprae* closely resembles

M. tuberculosis in its morphology. It is a slender rod sometimes as much as 6 μ in length. It is easily stained, non-motile, and does not produce spores or capsules. The organism is distinctly acid-fast, but stains somewhat more readily than *M. tuberculosis*. It has not been cultivated upon artificial media.

Disease Production. The disease resembles tuberculosis in some respects. It manifests itself in several forms. In some cases the principal symptoms are nervous. Certain portions of the body lose the power of feeling; they are said to become anesthetized. In other cases nodules resembling those of tuberculosis develop in the subcutaneous tissues. The disease frequently progresses slowly, the incubation period being extremely long, various estimates placing it at from three to twenty years.

Immunity. Practicable methods of immunization and treatment by bacteria or their products have not been developed.

Transmission. The means by which leprosy spreads is not certainly known. The organism is generally present in considerable quantities in the nasal mucosa of those who have the disease. It is not impossible that the nose is the infection atrium. Some investigators have believed the disease to be spread by mosquitoes, fleas, bedbugs, or other insects. The evidence is very inconclusive.

Treatment. Very few treatments have been of much value in curing leprosy, though some degree of success has been attained with intramuscular injections of chaulmoogra oil obtained from the seeds of various species of *Hydnocarpus*, a genus of tree found in Burma and other tropical countries. Diet and personal hygiene are also important. In recent years considerable success has been achieved by the use of some of the newer sulfa drugs and antibiotics.

Other Acid-fast Bacteria

It has already been noted that acid-fast bacteria are not uncommon in the intestines, soil, milk, etc. These resemble *M. tuberculosis* rather closely in their morphology, but develop much more luxuriantly upon culture media, particularly when the culture is kept at room temperature. It is sometimes impossible to differentiate between these organisms and *M. tuberculosis* except by animal inoculation, and even this method must be used carefully inasmuch as the injection of considerable quantities of normally non-pathogenic acid-fast organisms may cause in the tissues of susceptible animals a development of nodules somewhat resembling those of tuberculosis. Isolation of the organisms, however, from these nodules reveals distinct differences, so that in practice little difficulty is actually encountered. Acid-fast bacteria have been isolated from butter in a considerable number of cases. Except for the danger of confusion with the true tubercle bacillus, these organisms do not have any economic importance.

CHAPTER 41

The Ray Bacteria Group: The Genera *Actinomyces*, *Nocardia*, and *Streptomyces*

The order *Actinomycetales* (see Chapter 4) includes those bacteria that tend to produce long more or less branched filaments and through the development of a mycelium resemble the true fungi. The order is divided into three families. The first of these, the *Mycobacteriaceae*, which includes the acid-fast bacteria, has just been considered. The remaining two families resemble each other in that in all cases there is the rather definite organization of a mycelium as a mass of branched threads somewhat resembling those of the fungi, but much more slender. These families are differentiated on the basis of the tendency on the part of the mycelium to fragment into rods which apparently function as spores, or to remain without such fragmentation. The family *Actinomycetaceae* includes those forms in which the mycelium tends to fragment, the family *Streptomycetaceae*, those in which the mycelium does not show this tendency, but remains intact. In the latter family most of the species produce an *aerial mycelium*, that is, some of the threads grow up into the air and break up into spores. Three genera, two (*Actinomyces* and *Nocardia*) in the family *Actinomycetaceae* and one (*Streptomyces*) in the *Streptomycetaceae*, contain species that are of sufficient economic significance to warrant discussion.

The Genus Actinomyces

The first organism to be recognized and characterized, and now placed in this order of ray bacteria was *Actinomyces bovis* observed by Bollinger and described by Harz in 1877 in the lesions characteristic of the disease lumpy jaw in cattle. This organism was not cultivated until much later, but a great deal of confusion arose due to the fact that the organism is anaerobic and that organisms closely resembling it morphologically were readily isolated. These aerobic species belonging to the genera *Nocardia* and *Streptomyces* next to be discussed are quite abundant in nature and are grown so readily that their cultures were often mistaken for those of *Actinomyces bovis*.

Actinomyces bovis may readily be observed in the lesions of the disease lumpy jaw or actinomycosis. The pus from the lesions usually contains sulfur-colored granules. When these are examined microscopically they are found to consist of a tangled mycelium at the center with the ends radiating from the mass and with the tips enlarged and club-shaped. It was because of this arrangement of hyphae or threads that the names ray bacteria and *Actinomyces* were given. In culture the mycelium quickly fragments into bacillus-like segments which are gram-positive and non-acid fast.

Actinomycosis is a disease that is widely distributed wherever cattle are raised. Apparently the organism is a common inhabitant of the mouth of the bovine, where it grows on decaying food between the teeth. It has little power of primary attack, apparently must be introduced into wounds made by sharp objects such as the awns of certain grasses. It can grow in the necrotic tissue and under the anaerobic conditions there prevalent causes the development of an abscess, long-continued inflammation, and even a tumorous mass. Usually other organisms such as staphylococci are present in the lesion.

A disease somewhat resembling the actinomycosis of cattle has been recorded for man, caused by an organism very similar to that of lumpy jaw, named *Actinomyces israelii*. Some authors consider the two organisms to be identical.

The Genus Nocardia

The members of this genus differ from the preceding in being aerobic. The colonies on media develop first as a mass of branched hyphae; these tend to break up and the older colonies resemble those of mycobacteria. In some cases the cells are acid-fast, further emphasizing their relationship to the genus *Mycobacterium*. The mycelium is very slender, the diameter usually being between 0.5 and 1.0 μ. The mycelium at first shows no septa, but later cross walls form and the whole mycelium breaks up into rod-shaped or spherical cells which function as spores. In some species larger and thicker walled chlamydospores are formed; when these germinate they form several germ tubes. Waksman in the sixth edition of the Bergey *Manual* recognizes thirty-three species, and notes a hundred and one other names applied to species that have been inadequately described. Many of the species produce pigments.

The genus is made up largely of soil-inhabiting species. Thirteen are recorded from pathological conditions in man or animals. Several have assumed some importance as producers of antibiotics, as *Nocardia gardneri* from which proactinomycin is secured.

Nocardia farcinica was given as a name by Trevisan in 1889 to an organism that had been described by Nocard the preceding year. It is the cause of the disease bovine farcy, a cattle disease characterized by the development of enlargements and ulcers of the lymph vessels of the inner leg surfaces with later general involvement of lymph nodes. The disease is a chronic one, progressing for a year or more. In culture the filaments of the initial mycelium are very slender, about 0.25 μ in diameter and somewhat *acid-fast*. It grows readily upon culture media. The disease has been found in Europe but not in the United States.

Nocardia asteroides has been isolated on numerous occasions from cases of so-called pulmonary pseudotuberculosis in man and from other lesions such as abscesses. This organism resembles the preceding in being acid-fast.

Certain species, as *Nocardia madurae*, are found associated with Madura foot, a chronic infection of the foot characteristic of certain tropical countries.

The Genus Streptomyces

The genus *Streptomyces* is characterized by the development of a much-branched mycelium which produces branches which grow into the air (the aerial mycelium) and which function as conidiophores, producing spores from the tip or breaking up into segments to form spores. These conidiophores and their chains of spores are frequently characteristic in appearance, sometimes straight and sometimes curled into loose or tight spirals. Waksman (1948) recognizes seventy-three species and notes a hundred and sixty species names that have been proposed, but without descriptions adequate to make identification possible. Most of the species are from the soil. Great economic significance has come to certain members of the group because of their importance in the production of valuable antibiotics. Among these are *Streptomyces griseus*, producing *streptomycin*, *S. lavendulae*, *streptothricin*, *S. antibioticus*, *actinomycin*, *S. venezuelae*, *chloromycetin*, and *S. aureofaciens*, *aureomycin*.

Many of the species form pigments, some of which diffuse through the medium; others are apparently produced by the action of enzymes directly upon the constituents of the medium; and in some the pigment is confined to the mycelium and it does not diffuse.

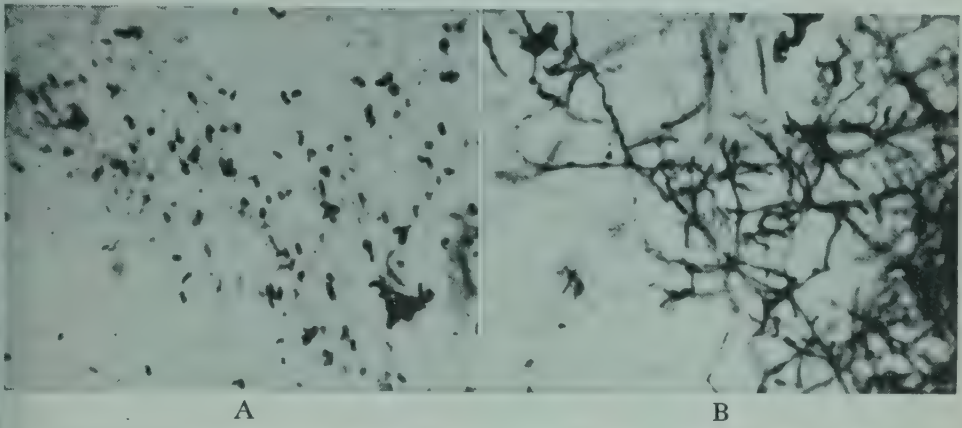

Fig. 41–1. *Streptomyces scabies*, the cause of potato scab. A. The spores produced by the aerial hyphae. B. The branched mycelium.

Several species of the genus are recorded as causing infections in man and animals. Some of them as *Streptomyces somaliensis* produce the hard fibrous swellings of the foot (known as mycetomata) found in the tropics, others have been isolated occasionally from other types of infections.

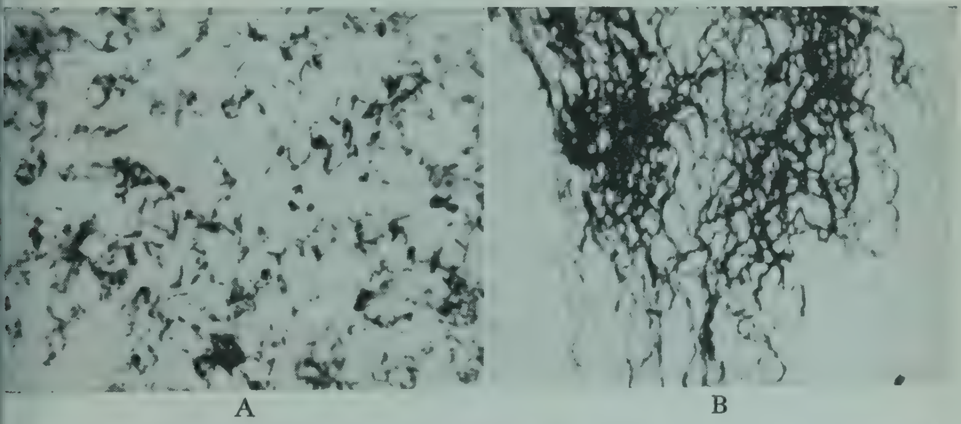

Fig. 41–2. *Streptomyces ipomeae* which causes a scab of the sweet potato. A. Chains of spores as produced by the aerial hyphae. B. Branched filaments of the vegetative mycelium.

Two species of *Streptomyces* produce important diseases of plants *S. scabies* is the cause of the destructive disease potato scab. This organism grows readily on artificial media, and is quite variable. The aerial mycelium is wavy or curved, with some spirals. The conidia produced are cylindrical, relatively large, 0.8–1.0 μ by 1.2–1.5 μ. A brown soluble pigment is produced. On agar there is an abundant cream-colored, wrinkled, raised growth. Gelatin is liquefied. The mycelium takes up the stain carbol-auromin and is readily demonstrated in the lesions of the potato by the use of the ultraviolet technique described for the tubercle bacillus. *S. scabies* has been found to parasitize a considerable range of host plants. *S. ipomoeae* produces a somewhat similar disease on the sweet potato.

CHAPTER 42

The Spirochete Group: The Genera *Treponema*, *Borrelia*, *Leptospira*

The bacteria of the order *Spirochaetales* differ from other bacteria in that the cell body is relatively slender, flexuous, and spiral. The bacteria are motile by means of a flexing movement of the cell or by rapid cell rotation about the long axis of the spiral. Structures resembling flagella have been observed in some cells by means of the electron microscope. Whether these also function is unknown. The cells of some forms are not readily stained by the usual laboratory dyes, but Giemsa stain is quite uniformly effective. Like other bacteria these organisms multiply by transverse fission, though some of the observations recorded in the earlier literature seemed to indicate longitudinal fission.

The order as recognized is made up of two families. The first, the *Spirochaetaceae,* includes a group of relatively large organisms from 30 to even 500 μ in length. The species of one genus, *Cristispira,* are parasitic in the crystalline style of certain mollusks; *Saprospira* and *Spirochaeta* are free-living forms found in water, the former in mud in salt water. The first species described, *Spirochaeta plicatilis,* was observed and named by Ehrenberg in 1838. The slender, actively motile cells are very long, 0.5–0.75 μ by 100–500 μ, with rounded ends. When later long spiral organisms were described as causing various diseases, they were placed by various observers in the genus *Spirochaeta,* and the casual designation "spirochetes" was given to all members of the group. Recognition that there were major differences in biology and morphology led to the suggestion by Schaudinn (1905) that the family *Treponemataceae* be recognized to include the much smaller, more predominantly pathogenic, flexile, spiral forms. Many of the species are parasitic in the blood stream or on mucous membranes. Three genera, *Borrelia, Treponema* and *Leptospira* contain important pathogenic species.

The Genus Borrelia

Murray and Davis (1948, Bergey's *Manual*) recognize seventeen species, all parasitic, and most as the etiologic agents of specific diseases. In addition one hundred and one names are listed of species insufficiently described. The organisms of this genus are usually from 8 to 16 μ in length, with the spirals somewhat coarse, shallow, and irregular, and with the ends tapering into fine filaments. Unlike the members of the other genera of the *Spirochaetaceae*, they stain readily with the ordinary aniline dyes.

The first species of this genus to be described was the etiologic agent of the disease European relapsing fever in man. It was observed in 1873 by Obermeier and named *Spirochaete recurrentis* by Lebert in 1874. It is now called *Borrelia recurrentis*. The organism is circular in cross section or slightly flattened, 0.35–0.5 μ by 8–16 μ, with pointed ends and terminal filaments. Culture is difficult, though growth has been said to occur in a medium of ascitic fluid and fresh tissue (such as kidney). The organism may be grown in the chick embryo. It is readily observed in preparations of the blood of infected individuals. The disease is characterized by its sudden onset, with chills and fever and headache. The spleen is moderately enlarged and tender. In three or four days the fever subsides, followed at varying intervals of several days to two weeks by relapses. The organisms are present in considerable numbers in the blood stream during the fever. Mortality from the disease is not high. The disease was originally recognized as louse-borne (*Pediculus vestimenti*) and so transmitted from man to man. Apparently the louse takes up the spirochete from the blood. The organism may be present in eggs laid by an infected louse. However the infection is not transmitted to man by the bite of the louse. When the louse is crushed its body fluids may enter through the bite, especially following scratching.

Since the discovery of the cause of European relapsing fever many other similar diseases have been identified. The causal organisms are usually indistinguishable from *B. recurrentis*, but have been given different names because of differences in locality, the vector, or the reservoir of infection. Whether they represent different species is quite uncertain. For the most part these relapsing fevers are transmitted to man by the bite of some bloodsucking arthropod, such as a tick, that has been infected by sucking blood from some animal which harbors the organism in its blood. *Borrelia duttonii* has been reported from Central African relapsing fever transmitted by a tick (*Ornithodoros moubata*), *B. berbera* from North Africa (Mediterranean coast) and *B. aegyptica* from Egypt. Several tick-borne relapsing fevers are known from North America, for the most part from the western and southwestern portions. *B. hermsi, B. parkeri,* and *B. tunicata* have been named largely on the basis of the species of tick transmitting the diseases. The exact relationship to the animal reservoirs of the disease has not been adequately worked out. It is probable that for the most part they are small mammals, usually rodents. Among the animals that have been infected with the different species of *Borrelia* are the mouse, rat, chipmunk, squirrel, fox, dog, opossum, armadillo, and porcupine.

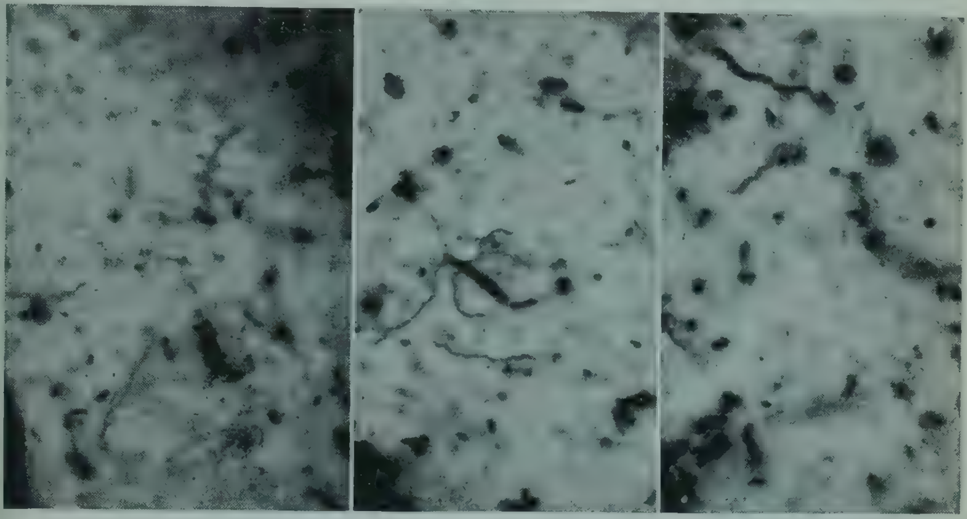

Fig. 42–1. Photomicrograph of stained mounts from the lesions of Vincent's angina. Note the several types of spirochetes present, also some fusiform rods.

Another species, *Borrelia vincentii,* is associated with a fusiform organism (*Fusobacterium plauti-vincentii*) in the disease called Vincent's angina or trench mouth. The disease is characterized by ulceration of the mucous membranes of the mouth, frequently with the formation of a fibrinous false membrane. The organism has not been cultivated. While generally associated with the disease, neither this organism nor the *Fusobacterium* has been shown to be causally related to it.

The Genus Treponema

The members of this genus differ from those of *Borrelia* in that they stain with difficulty with the usual aniline dyes, but satisfactorily by means of the Giemsa stain, or by use of the technique of impregnation with silver. The several species are believed to be strict anaerobes. In general morphology the cells resemble those of *Borrelia*. A terminal filament may be present.

Murray (1948, Bergey's *Manual*), in his revision of the genus recognizes eight species and notes as inadequately described about thirty-five others.

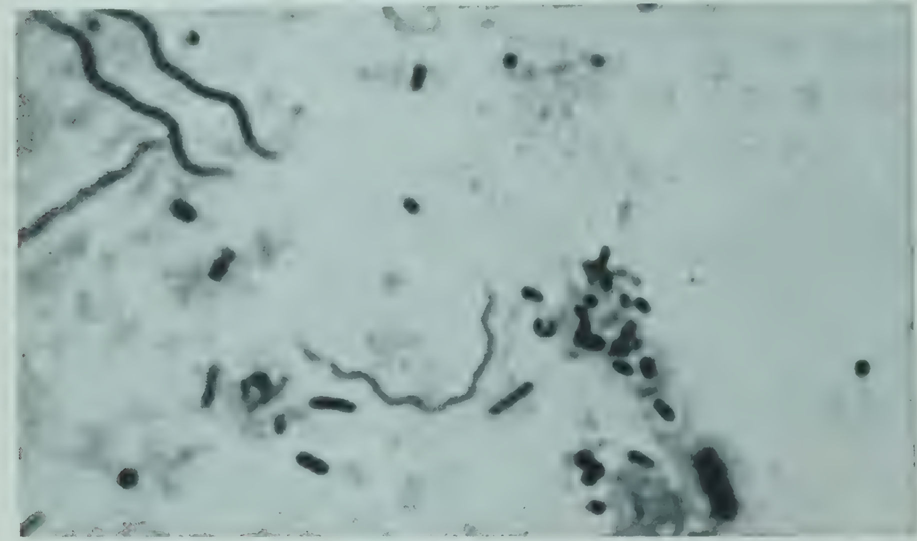

Fig. 42–2. Photomicrograph of spirochetes from the human mouth.

Four important diseases of man are caused by species of *Treponema:* syphilis, yaws, pinta, and bejel. The organisms causing the four diseases are practically indistinguishable, but the disease syndromes differ. The disease yaws is widely distributed in the eastern tropics, pinta in the tropical Americas, and bejel in the Arab countries. Syphilis is caused by *Treponema pallidum,*[1] yaws by *T. pertenue*, and pinta by *T. carateum*. The *T. pallidum* only will be considered.

Treponema pallidum

This organism is the specific cause of syphilis in man. It was first described by Schaudinn and Hoffman in 1905. Before that date many

[1] Generic names which end in *-ma* are usually from the Greek; they are neuter, hence the neuter form of *pallidum*. The plurals of such words end in *-mata*, as *Treponemata*.

organisms had at one time or another been described as the cause of the disease, but these are known now to be secondary invaders entirely. The disease is one which is widely distributed among civilized and semi-civilized peoples.

Morphology and Culture. *Treponema pallidum* is an exceedingly slender organism less than 0.5 μ in diameter and usually about 4–20 μ in length. The spirals are relatively regular and may vary in number from three to forty or even more. The organism is actively motile. The organism was not recognized earlier because of the difficulty experienced in staining it. This difficulty also accounts for our lack of knowledge regarding many of its morphological characters. One of the simplest methods for demonstrating it is to mix the organism secured from infected tissue, particularly the initial lesion or chancre, with specially prepared India ink upon a glass slide and allow this to dry in a thin film. The organisms do not take up the ink and may be recognized after the film is dried as transparent spirals in a dark background. When it is necessary to demonstrate them in tissues, complicated methods of staining are necessary.

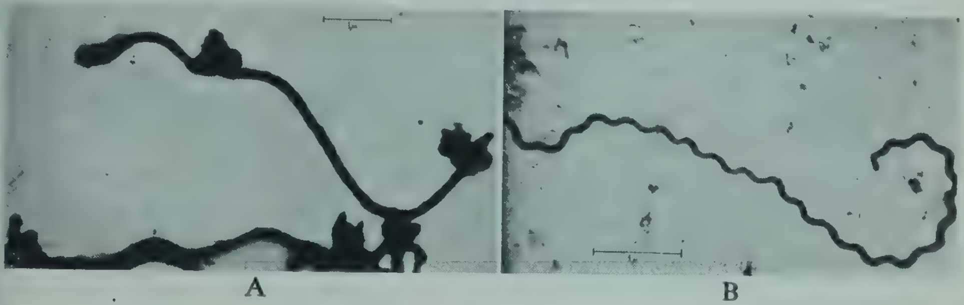

Fig. 42–3. A. *Treponema pallidum*. Electronograph. The cells of the spirochete are seen to be undulating, not very regularly spiral, with clusters of granules that may represent extrusions from the cell. Also note the wavy slime masses that resemble flagella. (Courtesy of H. E. Morton and T. F. Anderson and the *American Journal of Syphilology*.) B. *Leptospira icterohaemorrhagiae*. Electronograph. Note the close spirals and the ends of the cell to be hooked. (Courtesy of H. E. Morton and T. F. Anderson and the *Journal of Bacteriology*.)

The organism proved to be difficult of cultivation; in fact, there is real question as to whether it has ever been grown in laboratory media. Noguchi published what he believed to be a satisfactory method for securing cultures, but other workers have not in general been successful in its use.

Disease Production. The organism is readily found both in the primary and secondary lesions of syphilis and may sometimes be demonstrated in the tertiary lesions as well. The disease in a modified form may be transmitted to certain animals, particularly the rabbit and the anthropoid apes. The primary lesion in man is generally a chancre which develops in about three weeks after infection. The neighboring lymphatics are invaded and there is more or less enlargement of the lymph nodes. About six weeks after the appearance of the primary lesion the secondary lesions develop. It is probable that these arise from the general invasion of the blood stream by the organisms. There is marked skin eruption, more or less falling of the hair, and usually fever. The duration of these symptoms is variable. Sometimes they last for years. A relative immunity may be established, but eventually there may be tertiary symptoms such as degeneration of the liver and changes in the blood vessel walls, paresis, and locomotor ataxia.

Immunity. No method of conferring immunity has been developed. Treatment involves the use of salts of mercury, iodine, and certain organic arsenic compounds, arsphenamine and neoarsphenamine. Antibiotics have also proved useful. The disease is commonly diagnosed in the laboratory by means of the Wassermann complement fixation test and Kahn precipitin test.

Transmission. Syphilis is primarily a venereal disease, usually transmitted by direct contact. Infection has been traced in some cases to infective drinking vessels. Although in the strictest sense it is not inherited, it may be transmitted from one generation to the next. Children of infected parents are often syphilitic at birth (congenital).

The Genus Leptospira

The organisms belonging to this genus closely resemble treponemata, but are more readily cultivated in laboratory media. They are aerobic. There is also a tendency for the cells when growing in a liquid medium to show a semicircular hook at one or both ends. Terminal filaments are absent. Murray (1948) recognizes three species.

Leptospira icterohaemorrhagiae is the cause of Weil's disease, infectious jaundice, or leptospirosis in man. The causal organism was discovered by Inado and Ito (1914), closely followed by its identification in Europe, and since that time in practically all countries. The organism is primarily a parasite of the rat in which it may be demonstrated by examination of triturated kidney or of urine by use of the dark field. The proportion of wild rats infected in different localities has been found to vary greatly, as high as forty per cent in Japan. The leptospirae are discharged in the urine of the rat (or other infected rodent) and are present in sewage (sewer rats), mine water, and stagnant water. The disease is found in those who work in rat-infested mines, in sewage workers, and in those that come in contact with contaminated water. Just how the organism invades the body in man is not certain, possibly through the skin. After an incubation period of one to twelve days there is high fever, nausea, headache, and muscular pain. Jaundice appears in a large proportion (forty to sixty per cent) of the cases. The mortality may be as high as twenty-five per cent. Individuals who recover are immune. The organisms may early be demonstrated in the blood, but later tend to localize in the kidneys and are present in the urine.

A whole list of other leptospiroses have been described from various localities, with the host reservoirs various rodents as the field mouse, wood mouse, mole. It is uncertain how many distinct causal organisms there may be.

There is also a disease of dogs, sometimes called canine typhus, caused by *Leptospira canicola,* which may be transmitted to man.

CHAPTER 43

The Typhus-Tick Fever Group:

The Genera *Rickettsia*, *Coxiella*, *Cowdria*, *Bartonella*, *Miyagawanella*, *Colesiota*

The order *Rickettsiales* includes a group of microorganisms that resemble the true bacteria in many of their morphological characters, but are distinguished by the very strict parasitism of the cells. They grow only as intracellular parasites in or on the cells of animals and arthropods, possibly even protozoa. In general they are relatively minute; rod-shaped, coccoid, spherical, or somewhat irregular in shape. They are frequently somewhat pleomorphic, and are uniformly gram-negative. They often stain rather poorly by the aniline dyes of the bacteriological laboratory. The electron microscope seems to have demonstrated that these organisms have a structure including cell wall and protoplasm resembling that of bacteria. They have been cultivated in a few instances in media containing body fluids. They are non-motile (except in one genus). They are of considerable economic importance because of the diseases of man and animals which they produce.

The order is divided into three families largely on the basis of their pathogenic characteristics. The first family, the *Rickettsiaceae*, includes those forms which are found growing within tissue cells other than red blood cells and frequently cause disease in man and animals transmitted by means of arthropod vectors, such as lice, mites, fleas, and ticks. The second family, the *Bartonellaceae*, differs from the preceding in that the cells parasitized in the mammalian body are the erythrocytes. The diseases may also be transmitted by arthropods. The third family, the *Chlamydozoaceae*, includes intracellular disease-producing organisms that are *not* transmitted by vectors, but more or less directly by contact. Knowledge of these groups of organisms has grown rapidly within recent years, with the differentiation of many diseases that had long been confused and poorly understood.

The Family Rickettsiaceae

The organisms belonging to this family are small, frequently pleomorphic, usually bacillar, ovoid, or coccoid. They are particularly abundant in various arthropods such as lice, mites, and ticks. The ones of interest are those which are transmitted to man or from other animals to man by one of the arthropod vectors. None of them has been cultivated in media free of living cells. Three genera are recognized, *Rickettsia, Coxiella,* and *Cowdria*. Some of the more important diseases produced in man and animals by these organisms will be noted.

Rickettsia prowazekii is the cause of typhus fever (*typhus exanthematicus*). The disease has been endemic in parts of Russia and Poland and has spread in epidemic form during war, in concentration camps, and in prisons. It has been termed in consequence camp fever and jail fever. During World War I there were more than 300,000 deaths from this disease in Serbia. The organism is very minute, usually coccoid to short rods, occasionally filamentous. The coccoid forms are about 0.25–0.4 μ by 0.3–0.4 μ. It may be grown in the yolk sac of incubating eggs and in plasma tissue cultures. The disease is characterized by an incubation period of five days to two weeks; the onset is marked by severe headache, chills, and fever. A macular eruption appears on the skin about four days after the first symptoms and persists as long as there is fever. The acute symptoms disappear usually about the twelfth day, and recovery is rather slow. The death rate varies from about 5 per cent to as high as 70 per cent. The rickettsiae are to be found in the cytoplasm but not in the nucleus of the invaded tissue cells. The disease is spread by the human body louse, *Pediculus vestimenti*. The organisms may be demonstrated in the gut of infected lice. Under the name Brill's disease, typhus fever has become endemic in some of the larger cities of the eastern United States.

A vaccine may be prepared by growing the organism in the yolk sac of incubating eggs. The killed cultures are given as three doses at weekly or fortnightly intervals. It has been extensively and apparently successfully used in immunization in the armies of the United States.

Obviously the disease can be eradicated by elimination of the vector.

Diagnosis of the disease is greatly facilitated by the interesting fact that the blood serum of a person suffering from typhus will agglutinate quite specifically certain strains of *Proteus vulgaris*. This organism and the rickettsia possess some of the same antigen, though there is no reason to suspect any close relationship between *Rickettsia* and the *Proteus*. This agglutination test for typhus is named after the men who first described it, the Weil-Felix reaction. The *Proteus* strains used are usually designated as the x strains, and the strain most often used is ox_{19}.

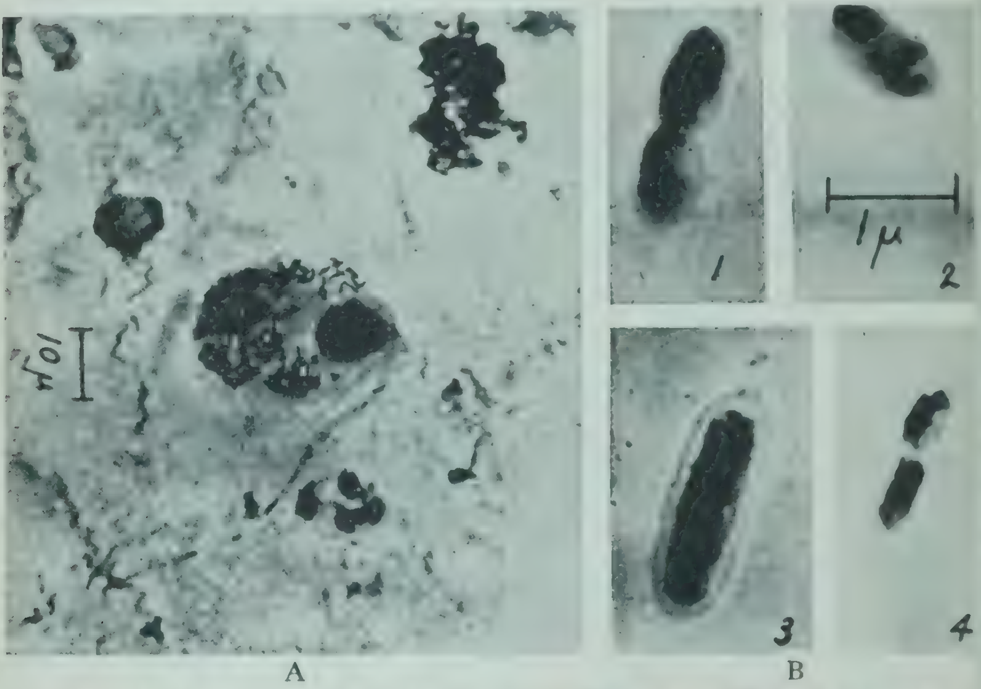

Fig. 43–1. A. *Rickettsia* of endemic typhus. Photograph. The rod-shaped organisms are embedded in the cytoplasm of an endothelial cell from the tunica of an inoculated guinea pig. (Courtesy of H. Plotz, J. E. Smadel, T. F. Anderson, and L. A. Chambers and the *Journal of Experimental Medicine*.) B. *Rickettsiae*. Electronographs of one or two organisms of several species. 1. Epidemic typhus, Breinl strain. 2. Endemic typhus, Wilmington strain. 3. Rocky Mountain spotted fever, Bitterroot strain. 4. American Q fever, American strain. (Courtesy of H. Plotz, J. E. Smadel, T. F. Anderson, and L. A. Chambers and the *Journal of Experimental Medicine*.)

Another important and very closely related disease is the so-called murine or rat typhus found in the United States and Mexico (where it is known as tabardillo), and widespread in many countries about the world. The infection reservoir is the rat, and the disease is transmitted among the rodents and to man by the rat flea and by the rat louse. When the disease is introduced into a louse-infested community, and is once established in man, it may become epidemic through spread by the body louse. The causal organism in murine typhus is *Rickettsia typhi.*

Rickettsia rickettsii (synonym, *Dermacentroxenus rickettsii*) is the cause of Rocky Mountain spotted fever, a disease which was first brought forcibly to attention because of its virulence in the Bitter Root Valley of Montana. It was later found to occur in a large portion of the area between the Cascade Mountains of Oregon and Washington and the central portions of Montana and Wyoming. Still more recently it has been found in the Mississippi Valley (Minnesota, Iowa) and in the eastern states, particularly in the mountainous districts. In most districts from which the disease has been reported it is relatively mild, the mortality being low. The disease is differentiated by the development of a characteristic skin eruption. It is a typical exanthema. Ricketts and his co-workers have shown that the disease is transmitted by the bite of certain ticks. It is probable that rodents, possibly also other animals of the regions where the disease is endemic, may harbor the organisms or be infected with the disease. In the laboratory it has been found to be transmissible to the guinea pig and to some other rodents. Ticks biting these animals, and later biting man, may transmit the disease to the latter.

The organism is extremely tiny (0.3–0.5 μ), pleomorphic, and gram-negative. It occurs abundantly in certain cells, frequently in the nuclei.

Those who recover from an attack of the disease show a marked immunity to reinfection. Passive immunization by use of blood serum from immune individuals has been practiced with some degree of success.

A somewhat similar disease called "fièvre boutonneuse" is endemic in some countries bordering on the Mediterranean, particularly Greece, Morocco, and Tunis.

Another group of rickettsial diseases is transmitted by mites. Of special interest is the *Rickettsia tsutsugamushi,* so named by the Japanese investigator Hayashi, from the Japanese name of the mite which is the active vector. The disease is widespread in Japan, Malaya, and the South Pacific islands. It was a matter of much concern to the medical staffs of the army during World War II. The disease reservoir is a field mouse, which is infested with a mite resembling somewhat the "chigger" mite of the United States. The larvae of the mite become infected through the egg from the infected mother. It is the larval mite that bites animals, including man, the adult mite apparently does not attack mammals.

The genus *Coxiella* includes forms that are very closely related to the true rickettsias, but differ in minor details. They are sufficiently small so that they may be termed filtrable, as they are not retained by Berkefeld N filters which hold back ordinary bacteria and Berkefeld filters which retain the rickettsias. The disease called Q (Queensland) fever is caused by *Coxiella burnetii*. The organism may be cultivated in tissue cultures and in the chick embryo. It was first recognized in Australia, and the natural reservoir found to be certain native animals of the bush. The disease is also present in the United States and probably is rather widespread in other countries.

Cowdria differs somewhat morphologically from the other rickettsias. The organisms are usually spherical or ellipsoidal. The single species is *Cowdria ruminantium*, the cause of heartwater disease of cattle, sheep, and goats in South Africa. The infection is highly fatal. Transmission is through ticks. The disease gets its name from the fact that large quantities of liquid accumulate in the pericardial sac, and in the pleural and abdominal cavities.

The Family Bartonellaceae

Four genera have been described in this family, *Bartonella, Haemobartonella, Grahamella*, and *Eperythrozoon*. Species that parasitize man have been found only in the genus *Bartonella*. The important diseases Oroya fever and verruga peruana seem to be endemic to a large area in mountainous western South America. The Oroya fever is characterized primarily by anemia, the verruga by an eruption of the skin. The organism is usually a motile rod with one to four polar flagella. Whether it is closely related to the other rickettsial groups is somewhat problematical. The organisms usually attack the red blood cells, causing a rapid destruction and the development of a severe anemia. If not fatal at this stage, the skin nodules appear. The disease is transmitted by sandflies of the genus *Phlebotomus*. What the native reservoir of the organism may be is not certainly known. Much difficulty has been found in working with the disease due to the relative insusceptibility of the laboratory animals.

The Family Chlamydozoaceae

Three genera have been described, *Chlamydozoon, Miyagawanella,* and *Colesiota.* The first two contain species that are pathogenic to man.

Chlamydozoon trachomatis is the name given to the organism described as the probable cause of the serious disease trachoma, an inflammation of the conjunctiva of the eye, not infrequently causing blindness. The organism is found inside the cells as so-called elementary and the initial bodies. The former are very small, 0.2–0.35 μ in diameter, they may be massed together to form larger bodies which may be 0.8 μ in diameter, and these in turn may form plaques as large as 10.6 μ in diameter. The organism has never been cultivated.

Organisms belonging to the genus *Miyagawanella* have been described as responsible for several diseases that have frequently been placed among those caused by filtrable viruses. Among these are the disease psittacosis or parrot fever (*M. psittacosis*), and the several so-called viral pneumonias of man, and lymphogranuloma venereum (*M. lymphogranulomatis*) which may manifest itself as the cause of genital lesions, inflammation of lymph nodes, meningitis, ophthalmia, and pneumonia in man.

CHAPTER 44

Viruses and Bacteriophage

The basis for differentiation and classification of the viruses (filtrable viruses) was discussed in Chapter 5. As noted there the only completely systematic classification which includes most of the forms which have been described is that of Holmes, published in the Bergey *Manual* (6th ed.). In the main, this will be followed. It is recognized that there are those who do not regard the viruses as living organisms and that their nomenclature should not conform to the usual biological rules. However, whether living or non-living, there must be names for their identification and for use in classification. There seems to be no good reason why the same general system of giving names should not apply here as with living things in general.

Bases of Classification. Can size and shape be used as a basis of classification? There is considerable difference in size between the largest virus particle and the smallest. Inasmuch as the viruses are measured in fractions of a micron, it is convenient to use as a unit of measurement the millimicron ($m\mu$), so defined that 1000 $m\mu = 1\ \mu$. The largest viruses are found in the poxes, such as smallpox, in which the particle is about 125–175 $m\mu$ in diameter, influenza 80–120 $m\mu$, fowl plague 60–90 $m\mu$, tobacco mosaic 33 $m\mu$, poliomyelitis 10–15 $m\mu$ and foot and mouth disease 8–12 $m\mu$. In fact, some of the viruses are almost as large as some of the rickettsias, others not much larger than some of the giant protein molecules such as that of hemoglobin.

The shape of virus particles may prove to be somewhat helpful. Many are approximately spherical, some, as that of tobacco mosaic, are cylindrical. Some of the bacteriophages have a spherical body with a straight or curved appendage or tail.

The necessity for using the electron microscope with attendant difficulties of making satisfactory preparations makes successful routine use of morphology as a basis for diagnosis rather problematical. Cultural characteristics of organisms that have not been cultivated obviously are ruled out, as are most physiological characters.

The only reliable present technique of identification seems to be to use the pathological changes that take place, the species of animal or plant attacked and the character of the lesion produced. The use of these criteria has given reasonably adequate differentiation.

We may regard the viruses as constituting a class *Vira* coordinate with the *Schizomycetes or* bacteria. The line of demarcation between the order *Rickettsiales* and the *Vira* is not too well defined; the two groups resemble each other in being obligate parasites of living cells.

The viruses are commonly divided into three major groups, those (suborder *Zoophagineae*) that parasitize animals, those (*Phytophagineae*) that parasitize plants, and those (*Phagineae*) that parasitize bacteria.

The number of viruses now known is so large that an adequate discussion would require a large volume and cannot be undertaken here. However, some species are so important that they should be considered in their relationships to others and to the diseases they produce.

THE VIRUSES INFECTING ANIMALS: ZOOPHAGINEAE

The viruses which produce disease in animal hosts have been divided into groups or families based primarily upon the type of disease produced, or in some on the nature of the host parasitized.

The first family (*Borrelinaceae*) includes the viruses that attack insects; the other five families include those which attack mammals and birds. These five include species which induce diseases of the type of the poxes, such as smallpox, and foot and mouth disease (the *Borreliotaceae*), the encephalitides in which effects on nerve tissues are significant, such as infantile paralysis and rabies (the *Erronaceae*), the diseases resembling yellow fever and influenza (the *Charonaceae*), the infectious anemia of the horse and fowl leucosis (the *Trifuraceae*), and the group of diseases related to mumps (the *Rabulaceae*). These will be discussed briefly.

Viruses Which Attack Insects: Family Borrelinaceae

The genus *Borrelina* includes six viruses, all of which attack the larvae of moths. One of these, *B. bombycis,* is of economic importance as the cause of a serious disease of silkworms called silkworm jaundice. About five days elapse after infection before symptoms develop, including yellow spots on the skin, and the appearance of the so-called polyhedral (many-sided) bodies in the blood. The disease is quite uniformly fatal. The other five species of the genus attack moth larvae which are highly destructive to plants of economic importance. For example, the caterpillar of the gypsy moth produces extensive damage by eating the leaves of shade trees. It is attacked and destroyed by the polyhedral virus *Borrelina reprimens*.

The disease honeybee sacbrood is caused by the single species of the genus *Morator* (*M. aetatulae*).

The Pox Viruses: Family Borreliotaceae

The virus diseases belonging to this group are generally characterized by the development on the skin of small discolored spots (macules), small circumscribed elevations of the skin with no fluid content (papules) or such with clear fluid contents (vesicles) or pustules.

The pox group of diseases is again divided into five subgroups of somewhat related diseases; the subgroup of the true poxes, including smallpox, fowl pox, and swine pox (genus *Borreliota*); the subgroup of the varicellas, including chicken pox and measles (genus *Briareus*); subgroup of the herpes diseases, such as so-called fever blisters and herpes simplex (genus *Scelus*); subgroup of hoof and mouth disease (genus *Hostis*); and subgroup of the wart diseases (genus *Molitor*).

The True Pox Subgroup. Of the three diseases belonging to this subgroup, fowl pox, swine pox, and smallpox, only the last with its etiologic agent *Borreliota variolae* will be discussed. Several distinct strains of the virus are known. *B. variolae* var. *hominis* is the virus of typical smallpox or variola in man, var. *bovis* is the vaccinia virus and cause of cowpox, and var. *ovium* is the virus of sheep and goat pox. There are two types of the disease in man, the dangerous malignant smallpox and a much less severe or benign type often termed *alastrim*. The death rate from the malignant type is high, 25 to 30 per cent, that from alastrim relatively low, less than 1 per cent.

So-called *inclusion bodies* found within the epithelial cells of pustules were first regarded as protozoa. Later, much smaller particles were described from the serum of vaccines. Probably the large inclusion bodies are compact masses of these smaller virus particles. The electron microscope shows these particles to be almost cuboidal shape and to have some granular internal differentiation. Typically there are five granules, one in the center and one in each of four corners. If suspended in 0.1 *N* NaOH the surface ruptures and the contents are extruded, the surface membrane remaining behind as a "ghost cell." This has been interpreted as a cell membrane surrounding several denser particles, not too different from descriptions of some of the rickettsias. The virus has never been cultivated in the usual laboratory media, but it grows readily on the chorioallantoic membrane of chick embryos.

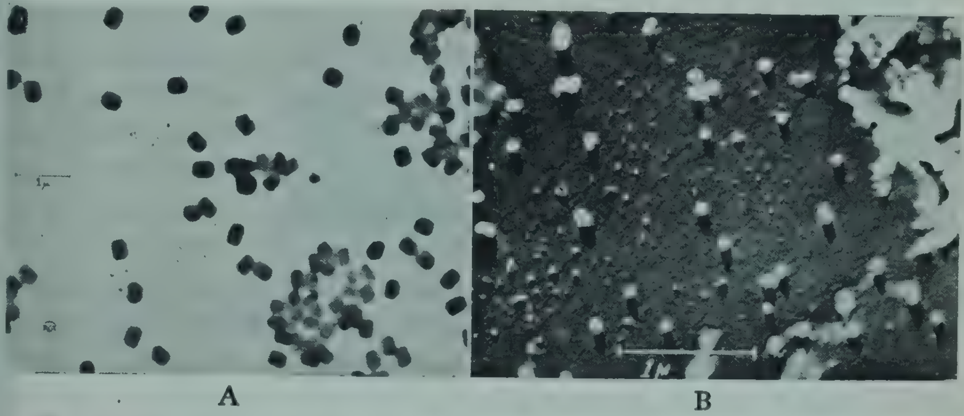

Fig. 44–1. A. Virus of Vaccinia. Electronograph. Note that the elementary bodies or virus particles tend to be rectangular in section, and that there is some evidence of internal differentiation. (Courtesy of R. H. Green, T. F. Anderson, and J. E. Smadel and the *Journal of Experimental Medicine.*) B. Virus of Human Influenza. Electronograph. The elementary bodies of the PR8 strain are seen to be spherical. This specimen has been shadow cast with chromium to bring the particles more sharply into relief. (Courtesy of R. C. Williams and R. W. G. Wyckoff and the Society for Experimental Biology and Medicine.)

Recovery from an attack of smallpox confers a relatively lasting immunity upon the individual. For several centuries at least vaccination against smallpox has been practiced. Originally it consisted of the inoculation into a healthy individual of the smallpox virus obtained from a pustule of a mild case of the disease. It was noted by Jenner in 1796 that milkmaids who contracted the disease cowpox from milking infected cows were relatively immune thereafter to smallpox. Vaccination against smallpox with lymph taken from cowpox pustules is commonly practiced at the present time, although the greater ease with which the virus may be grown in the chick embryo and the better control possible, have developed a trend to the use of chick embryo vaccine that may displace the use of calf lymph.

The latter is prepared with great care. Young heifers are usually chosen for its preparation. They are examined carefully to ascertain that they are entirely free from any infectious disease, washed, and kept in stables that can be thoroughly cleansed. The skin of the abdomen is shaved, then scarified by means of a curette, parallel lines being made over the greater portion. Lymph obtained from a previously vaccinated animal is then rubbed into this scarified area. Within several days (usually from five to seven) more or less inflammation will have developed in the skin and subcutaneous tissues, together with characteristic vesicles containing lymph. The contents of these vesicles are removed and mixed with 50 per cent glycerol. Tests are made by inoculation into guinea pigs to determine that there are no pathogenic organisms other than smallpox virus present. Particular care is used to exclude *Clostridium tetani*. This lymph is prepared for use by drawing it into capillary tubes which are sealed to prevent contamination. The organism retains its vitality under these conditions for considerable periods of time, and when rubbed into scarified skin, produces an infection termed *vaccinia*. In some cases the material used originally in producing pustules on the bodies of the calves came from cases of smallpox. The infection by scarification when properly performed rarely leads to serious results, and the individual becomes thereby relatively immune to smallpox. Vaccination is to be regarded as infection with an organism so attenuated that the disease will run a benign course.

Fowl pox, a virus (*Borreliota avium*) disease is troublesome in chickens and may infect other domestic fowl as turkey, duck, goose, and many wild birds.

The Varicella Group: The Genus Briareus

Two diseases in man, chicken pox and measles, are worthy of note. These diseases are characterized by reddened spots or rings which develop into vesicles or papules.

The virus of chicken pox (and herpes zoster) (*Briareus varicellae*) produces a highly contagious disease, usually in children. A vesicular eruption occurs in which elementary bodies are to be found. That they are specific for the disease seems to be indicated by the fact that they are agglutinated by the blood serum of convalescents. The disease is not transmissible to animals.

Measles (the virus *Briareus morbillorum*) is usually a disease of children, but may be epidemic among adults that have never been exposed. Such epidemics have occurred, for example, among isolated peoples such as the Eskimos or the South Sea Islanders. About a half million cases a year are reported in the United States. The disease begins with inflammation of the eyes and of the mucous membranes of the throat and with fever. This is followed by red spots on the mucous membranes of the mouth and the characteristic rash of the skin. Papules form, often crescent-shaped; on healing there is a brownish discoloration and desquamation (peeling). The virus has been grown in the embryo chick. The disease may be followed by secondary infections, such as pneumonia produced by streptococci or pneumococci.

The Herpes Subgroup. The Genus Scelus. Some seven diseases with their specific viruses are classified here. Most of them produce diseases in animals. One, herpes simplex, or herpes febrilis, affects man (virus *Scelus recurrens*). It is manifested by the appearance of an eruption of the skin or mucous membranes characterized by vesicles, often on the lips ("cold sores"). The virus particles are between 100–200 mμ in diameter. The virus may experimentally be transferred to laboratory animals and may be cultivated in the chick embryo.

The Foot and Mouth Disease Subgroup. The Genus Hostis. Foot and mouth disease (aphthous fever, aftosa) is widespread in parts of Europe and South America. It is primarily a disease of ruminants, particularly of cattle, but attacking sheep, goats, deer, and swine. It may experimentally be transmitted to the guinea pig, rabbit, and rat. There are a few authenticated instances of infection in man. In cattle the disease appears promptly after infection, usually within four days. There is fever, and vesicles appear on the lips, tongue, gums, and hard palate, and usually on the feet, between the toes, making the animal lame. There is abundant production of saliva. The animal cannot eat and loses in weight. The disease is not usually fatal, but nevertheless may occasion great losses. The disease has several times made its appearance in the United States, but has been stamped out by strict quarantine and by slaughter of exposed animals. The virus (*Hostis pecoris*) is one of the smallest; filtration studies seem to indicate a diameter of 8–12 mμ, centrifugation indicates 20 mμ. Several races or strains of the virus have been described, differing in virulence, and important because the immunity to one strain resulting from recovery from disease does not protect against other strains. Vaccines and immune serum may be used for immunization in areas in which the disease is enzootic. After World War II the disease was introduced into Mexico and spread rapidly over a considerable area. A quarantine line was established isolating the disease area, and an attempt was made to eliminate the disease from infected herds by slaughter. This proved ineffective because of owner opposition. Emphasis was then transferred to eradicating the disease by immunization, with maintenance of quarantine until an area has been "cleaned up."

A somewhat similar disease, vesicular stomatitis, occurs in the horse, caused by a distinct virus (*Hostis equinus*). The virus may be grown on the chorioallantoic membrane of the embryo chick. The disease has been reported from South Africa, the United States, and Europe. The virus particles are larger than those of foot and mouth disease.

The Wart Disease Subgroup. The Genus Molitor. Eight different viruses have been held responsible for the production of diseases which are characterized by the proliferation of tissue, usually of the skin, without the development of vesicles or of pustules. Two of these viruses have been found to produce infections in man, the common wart virus (*Molitor verrucae*) and the virus of molluscum contagiosum (*M. hominis*). The latter disease is characterized by the development of skin lesions at first like pimples which become small tumors with overlying skin stretched and shiny. Within certain cells of the skin may be observed relatively large particles termed the molluscum bodies, spherical or elongated, sometimes 30 μ or more in length. They are made up of a mass of elementary bodies about 0.3 μ in diameter, apparently the virus particles.

The Group of Encephalitis Viruses: Family Erronaceae

The viruses of this group are characterized primarily by their affinity for or tendency to grow in nerve and brain tissues. Three subgroups have been recognized, those viruses producing encephalitis ("sleeping sickness"), that is, producing lesions of the brain, and usually transmitted by arthropod vectors (genus *Erro*); the viruses of the poliomyelitis (infantile paralysis) subgroup, in which there is involvement of brain or spinal cord, the virus appearing in the feces (genus *Legio*); and the rabies subgroup in which there is involvement of nerve tissue only and transmission usually through bite of a rabid animal (genus *Formido*).

The Subgroup of Encephalitis Viruses. The Genus Erro. At least seven different viruses, some of them with several strains, have been found associated with encephalitis (sleeping sickness) of man and various animals. One of the first of the diseases definitely described was louping-ill, a disease of sheep found in Scotland and north England, occasionally transmitted to man. The virus (*Erro scoticus*) may be grown in the chorioallantoic membrane of the embryo chick. The vector is a tick, and transmission is from sheep to sheep. Some five different viruses have been described primarily from man; probably in all cases the disease is acquired through the bite of some arthropod vector, either ticks or mosquitoes. The encephalitis endemic in the United States (so-called St. Louis encephalitis, from the locality where first studied adequately) (virus *Erro scelestus*) is transmitted to man by the mosquito (*Culex tarsalis*). There is evidence that various wild animals and birds may constitute the reservoir of infection. Other diseases of this group are the forest spring encephalitis of Russia transmitted by a wood tick (virus *Erro silvestris*), Australian X disease (virus *Erro incognitus*), Japanese B. encephalitis (virus *Erro japonicus*), and the West Nile encephalitis (virus *Erro nili*). The disease equine encephalomyelitis in the United States (virus *Erro equinus*) affects horses and mules in many parts of North America, and is reported also from Argentina. The Borna disease in Germany is similar but apparently distinct. There are apparently two distinct strains in the United States, the Western and Eastern. The disease has been experimentally transmitted by mosquitoes and ticks. The disease caused by this virus has been found also in man—children are particularly susceptible. The virus increases rapidly in the chick embryo, causing death. Effective vaccines are prepared from infected embryos.

The Subgroup of the Poliomyelitis Viruses. The Genus Legio. The diseases of this group that have been differentiated are caused by at least six different viruses, four of them occurring in man, one in the mouse, and one in birds.

The most important of the diseases is human poliomyelitis or infantile paralysis (virus *Legio debilitans*). The disease is widespread. The prevalence of the virus is attested by the presence of specific antibodies in the blood of most adults. In spite of a vast amount of work, the disease is poorly understood. In part this is due to the difficulty in transmitting the disease to most laboratory animals except the monkey. The disease manifests itself most commonly in children, though it is not uncommon in adults. The characteristic symptoms are due to invasion of the brain and spinal cord. There may be sore throat, fever, vomiting, and more or less temporary or permanent paralysis of the legs or other parts of the body. The paralysis is due to the injury to or destruction of the large nerve cells found in the anterior horn of the spinal cord. It is from these that the motor fibers governing muscle motion originate, and their destruction results in paralysis. The virus particles are very small. The electron microscope shows them to be ellipsoidal and 20–30 mμ in diameter. The disease assumes epidemic proportions in many years during the late summer and early autumn. The method of transmission of the disease is not understood. There is evidence that the virus may enter through the throat, and pass by way of the nerves to the central nervous system. The virus is quite regularly present in the stools of those having the disease and frequently in those of healthy individuals that have been in contact with cases. It has been found in sewage. It is believed that the virus attacks primarily the membranes of the pharynx and intestine. Methods of immunization have not thus far been satisfactorily developed.

The Subgroup of Rabies Virus. The Genus Formido. Rabies (hydrophobia, lyssa), a disease involving the nervous system, is caused by a virus first studied intensively by Pasteur, who was unable to find a causal organism. It is now known to be due to a virus (*Formido inexorabilis*). All mammals so far as known and some birds may be infected. The disease has been reported from all parts of the world. The dog is most frequently infected. After an incubation period of a few days the animal acts strangely: there is a perverted appetite, and a tendency to bite, with consequent transmission of the virus, progressive inability to swallow, partial paralysis, and death. In man the disease usually shows a longer incubation period, even several weeks to two months. The disease is usually fatal if once the characteristic symptoms have developed. It is transmitted by the bite of the dog or of related wild animals, and in certain tropical countries, by the bite of the vampire bat which has been found sometimes to be infected in nature. In Mexico the disease, or one closely related under the name derriengue or bovine paralytic rabies, is said also to be transmitted by the vampire bat.

Negri, using stained sections showing the larger ganglion cells of the Ammon's horn of the brain, demonstrated the presence of specific bodies which have since been termed Negri bodies. There seems to be little question but that these bodies are quite constantly present in diseased animals and are not present in animals that do not have the disease, but it is not quite so certain whether they are specific organisms or are simply degeneration products. These Negri bodies in suitably stained preparations are found to vary in size from 0.5 to 25 μ in diameter. They generally show minute granular portions with inclusions of various kinds resembling chromatin granules. Negri believed these inclusions to be the cells of a protozoan parasite. The name which he proposed, *Neuroryctes hydrophobiae,* probably has priority and should replace *Formido.* The virus may be propagated in tissue culture and in the chick embryo.

The disease seems to be primarily one of dogs and related carnivora. It has been shown that the saliva of such animals is infective. The virus enters the body through wounds and passes along the peripheral nerves to the central nervous system. The portion of the central nervous system to which these nerves lead directly is in consequence the one usually most seriously affected.

A method of vaccination for rabies was developed by Pasteur. He found that the specific virus present in the spinal cord and brain of infected dogs shows considerable variation in virulence. His *fixed virus* is produced by repeated transfers of the disease from rabbit to rabbit until the virulence is sufficiently exalted so that the injections will kill an animal in from six to seven days. A rabbit is then injected with the fixed virus, and upon its death the spinal cord is carefully removed, using all aseptic precautions. This is then suspended in a desiccator over caustic potash and kept at a constant temperature of 23° C. in the dark for a week. The vaccine is prepared by emulsifying this cord. This drying process seems to attenuate the organism so that injections of the cord may be made without danger to the individual who has been injected, at the same time conferring a certain amount of immunity. Later other injections are made from cords that have been dried for shorter periods of time. The long incubation period of the disease gives an opportunity for repeated injections of material of this kind so that a considerable degree of immunity is established before the disease has had an opportunity to manifest itself, and the individual is protected.

Many other methods of preparing vaccines have been developed and are used in the prevention of the disease.

The Group of Yellow Fever Viruses: Family Charonaceae

The viruses of this group produce in man and animals diseases characterized by fever and by necrosis of tissues without special involvement of nerve cells and without the development of exanthemata (rashes and eruptions of the skin). Three subgroups are recognized, the typical yellow fever subgroup (genus *Charon*), the influenza subgroup (genus *Tarpeia*), and the hog cholera subgroup (genus *Tortor*).

The Subgroup of Yellow Fever Viruses. Genus Charon. Two diseases are included in the subgroup, that of true yellow fever (virus *Charon evagatus*) and that of Rift Valley fever, found in the so-called Rift of the eastern part of Africa (virus *Charon vallis*).

The disease yellow fever is one which usually is confined to tropical countries, but occasionally during warm seasons invades more temperate climates. It has been definitely shown to be due to a filterable virus. In some respects, yellow fever resembles an exceedingly virulent type of malaria, and like malaria it is transmitted by the bite of the mosquito and in no other manner. The mosquito most commonly responsible for the spread of the disease is *Aedes aegypti*. Several other species of *Aedes* as well as species of other genera have been found to be vectors in localities in Africa and South America. It is probable that, like the malarias, the virus of yellow fever passes through part of its life cycle in the body of the mosquito, for this insect does not become infective and cannot transmit the disease to another individual for a number of days after taking infected blood into its alimentary tract. The time which must elapse depends upon the temperature of the environment of the mosquito. The mosquito evidently serves as a true intermediate host.

The disease may be transmitted to the monkey and to some rodents. The disease may be stamped out by protecting yellow fever patients from the bites of mosquitoes and by preventing mosquitoes from biting uninfected individuals. Most important, probably, is the elimination of the breeding places of these mosquitoes. The recognition of the means by which this disease spreads has enabled the sanitarian to eradicate the disease from such cities as Havana in Cuba, where it had been known to be constantly present for a large part of the past century. This knowledge also renders very improbable any extensive invasion of our southern states, an invasion which has happened repeatedly in the past.

While these methods may be adequate in areas where the disease is not endemic, it has been found in recent years that in certain areas in South America and in Africa the virus persists in certain wild animals and may at any time be transmitted to man. This is the jungle yellow fever of Brazil. It is possible also that there is more than one type or strain of the yellow fever virus as evidenced by pathogenicity and cross inoculation studies.

Those who recover from the disease have a solid immunity. The virus may be grown in tissue culture, and from a suitably attenuated strain of the virus so grown, a vaccine reasonably satisfactory for use has been developed. Vaccination against yellow fever is a standard procedure for non-immune individuals that expect to visit tropical areas where the disease occurs.

The Subgroup of Influenza Viruses. The Genus Tarpeia. The viruses of this group are primarily responsible for inflammations of the respiratory system. Several infect animals primarily, the virus of cat distemper, of pneumoenteritis of calves, of dog distemper, of fox encephalitis, of ferret distemper, and laryngotracheitis of domestic fowls.

The disease influenza in man is an acute virus infection (*Tarpeia alpha* and *T. beta*) which has swept in great pandemics around the world in recent decades. It is much more abundant from November to March than during the remainder of the year. The ordinary form shows an onset of severe headache accompanied by pains and aches in the back, by fever, and by general prostration. The fever continues in such cases from three to five days and finally leaves the patient exhausted. The disease is often complicated by bronchitis or bronchial pneumonia. Immunity following recovery from influenza does not persist. Vaccines may be prepared from virus grown in chick embryos, but evidence of its usefulness is not conclusive.

Pfeiffer in 1892 described an organism which he believed to be the cause of the disease influenza. This was generally assumed to be the cause until later studies failed to prove any causal relationship between this organism and the disease. Finally the ferret in 1933 was found to be a satisfactory experimental animal and research became more productive. It seems to be demonstrated that the disease is caused by a filterable virus, but that the influenza bacillus (*Hemophilus influenzae*) may be an important associated organism. The virus may be recovered from nasopharyngeal washings and from nasal secretions.

The disease is apparently transmitted by inhalation of infectious droplets coughed out by those infected. The virus may be cultivated in the chick embryo. Suspensions of influenza virus from the allantoic fluid agglutinate red blood cells of the chicken. The electron microscope shows the virus particles to be round, bean- or kidney-shaped, with a central denser portion and about 80 mμ in diameter.

A closely related virus is the cause of the disease swine influenza. The virus alone causes only a mild reaction (termed the "filtrate disease"), but in presence of *Hemophilus suis* a serious disease is produced. The virus may be taken up by lungworms in diseased animals, and is transmitted to the eggs which are passed in the feces. These eggs are ingested by earthworms in which the larvae develop. The lungworms gain entrance when the earthworms are eaten by swine, and thus reintroduce the virus.

Perhaps the commonest disease affecting man, and one of the major causes of disability, is the common cold. Until the work of Dochez (1936) and others, the etiologic agent was not definitely fixed. It proved possible to transfer colds from man to the chimpanzee and from man to man by using nasal washings taken at the right stage of the disease. These nasal washings were filtered to remove all bacteria. The common cold is caused by a virus (*Tarpeia premens*). The virus of the common cold can be cultivated in a chick embryo medium, but repeated transfers lead to loss of virulence. Recovery from a cold apparently confers an immunity which soon disappears, and colds recur regularly. No method of immunization has been developed. There is no reasonable doubt that the common cold is an infectious disease readily transmitted from one person to another.

The Subgroup of Hog Cholera Viruses. The Genus Tortor. The viruses of this group involve many tissues of the body. They do not primarily attack the respiratory organs. Among the virus diseases produced are cattle plague or rinderpest, African horse-sickness, one type of mare abortion, blue tongue of sheep, panleucopenia of the cat, fowl plague, and Newcastle disease of fowls. Perhaps the most important from an economic point of view is the hog cholera virus (*Tortor suis*). The disease has long been known on the European continent, and is now almost world-wide in distribution. de Schweinitz and Dorset in 1903 proved that the etiologic agent is a filterable virus. Soon thereafter it was shown that the microorganisms, largely bacteria that had been thought to be causes of the disease were secondary invaders. The disease is highly fatal when it attacks a herd of hogs having no immunity. The disease may be acute or chronic. Upon autopsy the minute hemorrhages of the kidney surfaces giving a so-called turkey egg appearance are quite characteristic. The virus is present throughout the body, in the feces, and urine.

Transmission is usually by ingestion. The virus is quite minute, probably less than 35 mμ in diameter. Animals that recover possess a solid immunity. Such animals may be hyperimmunized by repeated injection of blood containing virus. The blood or serum from such hyperimmune animals may be used effectively through injection into susceptible animals to produce a passive immunity which is relatively short-lived. In practice it is converted into an active and relatively permanent immunity by vaccination of the animal with living virus.

The Group of Infectious Anemia Viruses: Family Trifuraceae

One genus only (*Trifur*) is recognized in this group of virus diseases characterized primarily by changes in the blood picture. One of them is infectious anemia of the horse, sometimes transmitted to man, reported from South Africa, Canada, Japan, and the United States, probably widespread. The virus passes a Berkefeld V filter (*Trifur equorum*). The second disease of this group (virus *Trifur gallinarum*) is fowl leucosis or range paralysis, widely spread and one of the most destructive of virus diseases. The disease is an extraordinary complex of lesions, so much so that many attempts have been made to differentiate two to several diseases. The disease can certainly be transmitted through the egg, and probably by contact.

The Mumps Virus: Family Rabulaceae

Only one subgroup is recognized (genus *Rabula*), including viruses showing special affinity for certain glandular tissues, particularly the salivary glands. Several types have been identified in the salivary glands of animals, including the guinea pig, hamster, rat, and mouse. The disease mumps or epidemic parotitis in man is caused by a virus (*Rabula inflans*) of this group. The disease in most cases involves the virus invasion of the parotid salivary glands, with swelling and pain; sometimes the submaxillary and sublingual glands are also involved. The virus is present in the saliva after two days. Occasionally the testes or the ovary may be involved, and rarely, inflammation of the meninges. Inclusion bodies 3–10 μ in diameter are to be found in the infected tissues. The disease is transferable to the rhesus monkey. The virus of mumps from the gland of an infected monkey may be propagated within an embryonated egg. Transmission of the disease is probably by infectious droplets. Solid immunity follows recovery from an attack of the disease. Passive immunization using the serum of immune individuals is not well demonstrated; doubtful.

THE VIRUSES AFFECTING PLANTS: PHYTOPHAGINEAE

The diseases of higher plants produced by viruses are too numerous to permit of any extended discussion. The Holmes (1948) classification includes six families with sixteen genera and one hundred twenty-eight species. Almost exactly half (sixty-seven) are included in one genus, *Marmor*, the mosaic group. No other genus includes more than nine species.

Certain of the plant viruses have lent themselves rather more satisfactorily to study of their chemical and physical characteristics than have the animal viruses. By maceration of virus-containing tissues of certain plants the particles may be obtained in a colloidal suspension mixed with various other materials from which they may be separated by precipitation by salts such as ammonium sulfate, by electrophoresis, by adsorption on certain substances such as kaolin, celite, or bone charcoal and from which they may be removed by suitable means. In other words, it has proved possible to purify certain viruses satisfactorily. Stanley has shown that in some cases the virus particles may form crystals.

Apparently none of the plant viruses has been grown independently of living plant tissues.

Most of the plant viruses are transmitted from plant to plant by insect vectors, usually by sucking insects of the *Homoptera* or *Hemiptera* or by thrips (*Thysanoptera*). In some the method of transmission is unknown. Artificial inoculation in a few cases has been solely by grafting diseased tissues on healthy plants.

The plant viruses have been classified in many ways. It is possible a classification based upon the morphology of the particles might be developed, but no adequate studies have been completed that would give any hint as to the usefulness of such procedure.

Practically, the classification of the viruses must perforce be limited to a relatively few characters. These are:

1. The species of plants attacked.
2. The specific lesions produced.
3. The methods of transmission, particularly the kinds of vectors.

The kinds of lesions produced and the vectors have been used by Holmes for the separation of six families as follows:

Type of Disease	*Type of Vector*	*Name of Family*
1. Yellows	Leaf hoppers	*Chlorogenaceae*
2. Mosaics	Aphids	*Marmoraceae*
3. Ringspots	Unknown	*Annulaceae*
4. Leaf curls	White flies	*Rugaceae*
5. Crinkling of foliage	True bugs	*Savoiaceae*
6. Spotted wilts	Thrips	*Lethaceae*

A few illustrative types of plant virus diseases will be noted.

Aster Yellows. Synonyms. Lettuce white heart. Fleabane yellows. Virus *Chlorogenus callistephi.* The disease gets its name from the China aster, the host on which the disease was originally observed and on which it has been most studied.

The virus is remarkable for the number of kinds of plants in which it can produce disease. More than a hundred twenty species distributed among thirty-eight different plant families have been shown to be susceptible. Some of these are plants of economic significance, and the losses from disease may be severe. Among the susceptible plants are lettuce, carrot, endive, celery, buckwheat, and parsnips. However, not all plants are susceptible, no species of the bean family (*Leguminosae*) has been infected. The symptoms of the disease are not identical for all hosts, but usually the veins of the leaves become cleared, and all new growth is yellowish (chlorotic) rather than green. Dormant buds start growth, the branches are unusually erect, there is malformation, flowers are virescent, and frequently do not set seeds. The disease is not transmitted through the seed. Plants cannot be infected by inoculation of virus mechanically, but can be infected by grafting. Usually the insect vector transmits the disease. The virus concentration in the leaf hopper vectors increases rapidly after feeding on diseased plants, as much as one hundred times. Two methods of controlling this infection may be through use of insecticides to kill the vectors or by breeding of resistant plants. Much progress has been made, for example, in breeding asters highly resistant to infection.

The genus *Marmor* (the mosaic viruses) as noted above includes about half of all plant viruses known. The tobacco mosaic virus (*Marmor tabaci*) has probably been the most studied. It was the first disease shown to be due to a filterable virus, by Iwanowski in 1892. It is a disease primarily of tobacco, but may be transferred to other species of the family *Solanaceae*. The disease in tobacco usually manifests itself as yellowish green areas in the leaves, clearing of the veins and green-yellow mottling of young leaves which may be malformed. In contrast to aster yellows just discussed, this disease is very readily transmitted. Juice from an infected plant placed upon an abrasion in a healthy plant will induce the disease. Or it may be transferred by inoculation of virus suspension by the hypodermic needle, by certain aphid insect vectors, by grafting, through the soil and even through the stems of dodder parasitizing both diseased and healthy plants. Ripe tobacco seeds do not contain the virus, and apparently it is not present in pollen grains. Antisera prepared against this virus show cross reactions with all strains of this virus that have been studied. Electronographs show the virus particles to be 10–20 mμ in diameter and to average 270 mμ in length. The virus can be secured in quantities sufficient for chemical examination. The particles are largely nucleoproteins. Analysis has shown the percentages of the following amino acids to be: tyrosine 3.9, tryptophan 4.5, proline 4.6, arginine 9.0, phenylalanine 6.0, serine 6.4, threonine 5.3, cysteine 0.68, alanine 2.4, aspartic acid 2.6, glutamic acid 5.3, leucine 6.1, valine 3.9, nucleic acid 5.8, and amide nitrogen 1.9. Some carbohydrate is present. The particles may be flocculated out from suspensions by salts such as ammonium sulfate used for protein precipitation.

A related virus (*Marmor dodecahedron*) produces the disease bushy stunt of tomatoes. It may be transmitted to other *Solanaceae* and to some other plants as the bean. It has been reported primarily from Britain. The name is derived from the characteristic manifestation of the disease in older plants, the growing tips die or cease growth, the axillary buds develop to produce a bushy top. The virus is noteworthy because of the nature of the virus particles. They are approximately spherical and 20–30 mμ in diameter. Purified suspensions of the virus will crystalize from solutions of ammonium sulfate as twelve-faced crystals (rhombic dodecahedra) which have the peculiar property of shrinking and swelling on drying and wetting.

Among the additional virus diseases of plants that are of economic significance in the United States the following may be listed, together with their virus names: potato witches'-broom (*Chlorogenus solani*), cranberry false blossom virus (*C. vaccinii*), peach X-disease (*Carpophthora lacerans*), peach rosette (*Carpophthora rosettae*), alfalfa dwarf (*Morsus suffodiens*), elm phloem necrosis (*Morsus ulmi*), potato yellow dwarf

(*Aureogenus vastans*), peach-wart (*Galla verrucae*), sugar-cane chlorotic streak (*Fractilinea quarta*), bean mosaic 4 (*Marmor laesiofaciens*), tobacco etch (*Marmor erodens*), cucumber mosaic (*Marmor cucumeris*), potato mild mosaic (*Marmor solani*), potato aucuba mosaic (*Marmor aucuba*), celery mosaic (*Marmor umbelliferarum*), cauliflower mosaic (*Marmor cruciferarum*), turnip mosaic (*Marmor brassicae*), sugar beet mosaic (*Marmor betae*), lettuce mosaic (*Marmor lactucae*), dahlia mosaic (*Marmor dahliae*), bean mosaic (*Marmor phaseoli*), pea mosaic (*Marmor leguminosarum*), alfalfa mosaic (*Marmor medicaginis*), tulip color adding (*Marmor tulipae*), iris mosaic (*Marmor iridis*), sugar-cane mosaic (*Marmor sacchari*), onion yellow dwarf (*Marmor cepae*), red clover vein mosaic (*Marmor trifolii*), cowpea mosaic (*Marmor vignae*), pea wilt (*Marmor repens*), alsike clover mosaic (*Marmor fastidiens*), pea mottle (*Marmor efficiens*), wheat mosaic (*Marmor tritici*), brome grass mosaic (*Marmor graminis*), rose mosaic (*Marmor rosae*), apple mosaic (*Marmor mali*), strawberry crinkle (*Marmor fragariae*), strawberry yellow edge (*Marmor marginans*), red raspberry mosaic (*Marmor rubi*), peach mosaic (*Marmor persicae*), cherry mottle leaf (*Marmor cerasi*), peach line pattern (*Marmor lineopictum*), cherry banded chlorosis (*Marmor pallidolimbatus*), cherry vein clearing (*Marmor nerviclarens*), dodder latent mosaic (*Marmor secretum*), celery calico disease (*Marmor aevi*), radish mosaic (*Marmor raphani*), primrose mosaic (*Marmor primulae*), fig mosaic (*Marmor caricae*), potato spindle tuber (*Acrogenus solani*), potato leaf roll (*Corium solani*), sugar beet yellows (*Corium betae*), raspberry leaf curl (*Corium rubi*), raspberry decline (*Corium ruborum*), loganberry dwarf (*Nanus loganobacci*), raspberry streak (*Nanus orientalis*), peach phony disease (*Nanus mirabilis*), strawberry witches'-broom (*Nanus fragariae*), strawberry stunt (*Nanus cupuliformans*), prune dwarf (*Nanus pruni*), flowering cherry rough bark (*Rimicortius kwanzoni*), citrus psorosis (*Rimicortius psorosis*), pear stony pit (*Rimicortius syri*), tobacco ringspot (*Annulus tabaci*), tomato ringspot (*Annulus zonatus*), tobacco streak (*Annulus orae*), broad ringspot of tobacco (*Annulus apertus*), potato mottle (*Annulus dubius*), tobacco leaf curl (*Ruga tabaci*), cotton leaf curl (*Ruga gossypii*), sugar beet curly top (*Ruga verrucosans*), beet savoy disease (*Savoia presmae*), tomato spotted wilt (*Lethum australiense*).

THE VIRUSES AFFECTING BACTERIA: PHAGINEAE

The existence and some of the characteristics of the bacteriophages have previously been discussed. They constitute a group showing so many points in common with the viruses that they are included in the class *Vira*.

A French investigator d'Herelle in 1916 began the publication of a series of papers and monographs which laid the foundation for much of our present knowledge of these viruses. He found that from feces, sewage, and other sources it was possible to secure cultures of an ultramicroscopic particulate substance which when mixed with young cultures of bacteria caused them to undergo lysis. For example, when he filtered a suspension of fecal matter through a porcelain filter with pores fine enough to retain bacteria and added the filtrate to a young culture of dysentery bacilli, the latter would soon pass more or less completely into solution. In broth cultures of bacteria the cloudy suspension would be cleared and the bacteria disappear; in growth on the surface of media clear areas would develop in which the bacteria had been dissolved. d'Herelle was convinced that he was dealing with an ultramicroscopic organism parasitic on the bacterial cells and gave to the supposed organism the common name of bacteriophage and the technical name of *Bacteriophagum intestinale*.

Investigators in many places took up the study of the phenomenon and rapidly added to our knowledge. Even yet, however, there is no complete agreement as to the exact nature of the cause. One group agrees in general with d'Herelle that bacteriophage is an ultramicroscopic organism which grows on the bodies of bacteria and destroys them, in fact, produces a fatal type of virus disease of the bacterial cell much as the bacteria produce diseases of higher plants and animals. When a suspension of bacteria thus parasitized is filtered through a bacteria-proof filter, the filtrate is found to be able to induce lysis in other bacterial cultures of the same species into which it is introduced.

A second group of investigators is inclined to the belief that bacteriophage is not an ultramicroscopic organism but something of the nature of an enzyme. It is suggested that the bacterial cells when attacked by this enzyme or lysin are destroyed, but that in the process of cell digestion there are produced greatly increased quantities of the lytic material. In other words the lysin or phage catalyzes its own increase just as certain chemicals catalyze their own increase in a chemical reaction. For example, the ester, ethyl acetate, hydrolyzes more rapidly in an acid than in a neutral solution. The addition of an acid increases the rate at which it breaks down into alcohol and acetic acid. As it decomposes it produces more and more acid which in turn increases the rate of the breakdown. A similar type of autocatalysis has been suggested as a logical explanation of the increase of phage without the necessity of regarding it as the result of the growth and multiplication of an organism.

Unity or Multiplicity of Bacteriophages. It was early found that many distinct bacteriophages may be demonstrated. The phage which actively attacks the typhoid bacillus will not affect the staphylococcus. Inasmuch as phages have been isolated for a great many species of bacteria, it is evident that there must exist a multiplicity of specific bacteriophages. But this is not the whole story. There is reason to believe that not all the phages isolated for one and the same species of bacterium are identical. The rapidity of lysis, the size and transparency and shape of the plaques formed by the several phages may differ widely. Furthermore, some of the phages may be shown to be not only species specific but strain specific. If a number of different phage isolations are tested on a number of different strains of the same bacterial species, marked differences will be found. Some may attack one strain only, others a group of several strains. It may even happen that there may be partial but not complete overlapping of the strains attacked. No serious attempt has thus far been made to classify the bacteriophages. It is evident, however, that they can be demonstrated in great numbers.

Methods of Isolation and Demonstration. Various methods have been proposed and used for the isolation of bacteriophages. One of the most commonly used is to add some sewage to a broth culture of the bacterium for which it is desired to isolate a phage, incubate, and filter through a porcelain filter to remove all microscopic organisms. When a small amount of this filtrate is then added to a pure culture (young) of the specific bacterium, lysis will usually occur. This does not, of course, prove that one kind of phage and one only has been secured. Several types may be together producing lysis. It has been possible to purify and isolate the different phages by a technique that resembles the method used for purifying mixed cultures of bacteria by plating. In this case a series of dilutions of the phage filtrate is prepared and mixed with broth suspensions of the homologous bacterium. This is spread over the surface of nutrient agar plates. The bacteria tend to grow as a uniform film over the surface. Wherever a particle of phage occurs it will produce a lysed area (called a *plaque*) in the bacterial film. Some of the dilutions should be such as to give well-isolated plaques. Transfer from such a lysed area should give pure phage. In some cases repeated platings may be necessary.

Whether or not the phage is pure may be determined in part by the uniformity in the characteristics of the plaques produced.

Classification of Bacteriophages. Holmes has proposed the single genus *Phagus* with the type species *Phagus minimus* the bacteriophage S_{13} of *Escherichia coli.* He recognizes forty-six species, differentiation being based upon size of particle, size and character of plaque produced, and species and strain of bacteria attacked.

Size and Morphology of Bacteriophage Particles. The method of isolation described above is evidently dependent upon the particulate nature of the bacteriophage. The success of the method seems to constitute good proof. Phage particle size as determined both by use of filters and of the electron microscope varies from the 8–12 $m\mu$ of *Phagus minimus* to the 50–75 $m\mu$ of *Phagus maximus*. Some bacteriophages are spherical or ovoid. Many have a tail-like appendage somewhat less in diameter than the "head," and in length several times the diameter of the head.

Nature of the Action of Phage. The exact manner in which the phage acts upon the bacterial cell is not well understood. The work of Bronfenbrenner and his co-workers indicated that the cells apparently disintegrate suddenly, almost explosively, and as though some stimulus had produced a high internal pressure in the cells with consequent rupture. The burst of a parasitized cell of *Escherichia coli* releases about two hundred phage particles.

The bacteriophages are somewhat dependent on their nutritional environments, exclusive of the bacterial cell. The phage particle must be adsorbed to the bacterial cell before it can become active and increase. So-called *cofactors* have been discovered for certain strains of phage, which apparently function. For example, for certain phages the presence of the amino acid tryptophan is necessary before the phage can be adsorbed and become effective. It has been suggested that bacterial mutants resistant to the attack of a phage strain which lyses the parent strain may owe their non-susceptibility to loss of ability to excrete enough of the cofactor needed, or there may be some difference in the bacterial cell which makes the cofactor unavailable.

Different strains of phage show differences in their virulence. In some cases apparently the phage does not destroy the cells but modifies them, even though they continue their growth. It has been suggested that some of the mutations of bacteria are induced through the parasitism of the phage.

In some cases the phage fails to destroy all the bacterial cells in a culture, and some resistant cells persist and multiply, giving rise to a strain showing more or less marked resistance to the attack of the phage. By appropriate techniques it is possible to induce mutation of phage and the development of strains having new or different characters.

Bacteriophage is readily destroyed by heat, a temperature of 65° to 70° C. usually being sufficient, while d'Herelle (1917) states that all races of bacteriophage are completely inactivated in thirty minutes at 75° C. Most races are very resistant to low temperatures.

Inasmuch as bacteriophage is non-toxic to man and animals, it has been suggested that it might be utilized in the treatment and cure of disease. Many efforts have been made in this direction, and some favorable reports of this method were recorded. However, the evidence in favor of such utilization of bacteriophage is not particularly convincing, and phage is not used therapeutically.

Pure strains of phage may be used for the differentiation and identification of specific strains or species of bacteria in a manner analogous to the use of the various immune sera such as agglutinins and precipitins. This has proved practicable, and methods of "typing" cultures of pathogenic bacteria are being developed rapidly. For example, Fisk and Madvin (1944) found it possible to type seventy-eight strains of staphylococcus from thirty patients by use of the phage susceptibility method. The phage types did not correlate with chromogenesis of the bacteria, their ability to hemolyze, or their production of toxin.

Some phages have proved to be important economically through their destruction of desirable bacteria. Elizabeth McCoy found that difficulties and inefficiencies in the commercial fermentation of molasses were due to the presence of destructive bacteriophages which parasitized the butyl bacteria.

Nelson and his associates have also determined that one of the major problems in the manufacture of cheese from pasteurized milk is the inactivation of the bacteria of the starter used by bacteriophage.

CHAPTER 45

Fungi and Yeasts Pathogenic for Man and Animals

A number of fungi, including yeasts, are known to produce disease in man or animals. The organisms producing disease are scattered among the various groups of fungi, though most of them belong with the imperfect fungi (*Fungi Imperfecti*). It is not practical here to discuss in detail all the species of economic significance because of their pathogenicity. A few which are representative of the group will be briefly noted, including only forms producing disease in man or animals, not plants. A disease caused by a fungus is called a *mycosis* (pl. *mycoses*). Names of fungus diseases affecting parts of the body have been coined by adding *mycosis* to the name of the tissue or organ involved, as *dermatomycosis* for a fungus infection of the skin, *pneumonomycosis* for a lung infection, *otomycosis*, fungus infection of the ear; in some cases the disease is named by suffixing mycosis to the stem of the generic name of the causal fungus, as in *coccidioidomycosis*. The pathogenic fungi and certain pathogenic yeasts will be considered in order.

The Genus Coccidioides

One species of this genus has been described, *Coccidioides immitis*. It is variously named valley fever, coccidioidal granuloma, and commonly *coccidioidomycosis*.[1] The disease manifests itself in man in one of two forms. Usually it is a relatively mild mycosis of the respiratory system which heals spontaneously. More rarely the disease becomes chronic and spreads throughout the body, causing lesions of the type called granulomata, and giving the name coccidioidal granuloma to the disease.

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[1] A good example of poor word formation. The name means literally a fungus disease caused by *Coccidioides*. However, the latter is the generic name of a fungus. It seems superfluous to coin a word meaning fungus disease caused by a fungus. *Coccidioidosis* might have been better etymology.

The organism when observed in the tissue superficially resembles the intestinal protozoan genus *Coccidium* in that a number of spores are produced within the cell. The generic name *Coccidioides,* meaning "resembling *Coccidium,*" is descriptive even though its author thought he was describing a protozoon. The causal organism was first described from the severe and highly fatal type of the disease, and this was the only source known for more than forty years. The disease was described first from Argentina, and in 1894 from California. Then beginning with 1937 a series of studies by Dickson and by Gifford showed that a relatively benign but widespread disease known as valley fever or desert rheumatism was caused by the same organism. It is now clear that the disease rarely if ever is transmitted directly from person, but that the infection arises from the inhalation of wind-blown dust in those southwestern parts of the United States which are semiarid. New cases in California do not commonly develop during the rainy season.

The causal organism exhibits a very different appearance in the characteristic lesions in the body from that in culture media. The spherical spores of the fungus in the body grow in size from an initial 1–4 μ in diameter to 20–60 μ, occasionally 80 μ in diameter. Eventually the protoplasm in the cell is divided by cleavages which originate in the outer layers and extend toward the center, this process of division and subdivision continuing until the whole interior is filled with many small spores. The process is not unlike that which has been detailed for the production of spores in *Mucor* and *Rhizopus*. The sporangium breaks and the spores infect neighboring tissue and repeat the cycle. Noteworthy is the fact that there is no formation of hyphae and no budding. There is some resemblance to certain of the parasitic protozoa, and the early identification of the organism as a protozoan is understandable. That it is a fungus, however, becomes at once apparent when the organism is grown in culture. A cell at any stage of development, whether spore or developing sporangium, germinates at once and produces an abundant mycelium which in about eight days produces numerous spores of a character quite different from those of the sporangium. The aerial hyphae develop lateral specialized enlarged branches which develop into chains of spores, probably to be regarded as chlamydospores. These spores are readily blown about and have been the cause of infection of those working with the cultures in the laboratory. When transferred to fresh culture medium the spores produce the fungus type of growth, but when injected into a suitable laboratory animal these spores develop into typical sporangia.

There develops in suitable liquid cultures of the organism an antigenic substance analogous to tuberculin. This is termed coccidioidin and constitutes a reasonably accurate diagnostic agent. A skin test is made by injecting 0.1 milliliter of a 1–1000 dilution into the skin. Redness and swelling in forty-eight hours constitutes a positive reaction. Both those infected and those who have had the infection at some time in the past give the reaction. A large proportion of those resident in certain areas of the southwestern portion of the United States give a positive reaction.

Initial coccidioidomycosis which results from inhalation of the spores usually remains as a focal infection in the lung. The disease frequently is without symptoms. In some cases there are definite symptoms such as pleurisy or pains in muscles and joints. Recovery usually occurs. In a few cases, a small fraction of 1 per cent, the organism is disseminated to many parts of the body, closely simulating tuberculosis. Such extensions are serious, frequently fatal.

FUNGOUS SKIN DISEASES: THE GENERA MICROSPORUM, TRICHOPHYTON, AND EPIDERMOPHYTON

Several genera and species of fungi are known which produce diseases of the skin. Such a fungus-caused disease may be termed a *dermatomycosis* (literally, skin fungus infection) or perhaps more commonly but less appropriately as a *dermatophytosis* (literally, skin plant infection). Several genera of the fungi and numerous species have been described, with three genera, *Trichophyton*, *Epidermophyton*, and *Microsporum* most significant. These organisms attack the skin and hairs, not other parts of the body. Their names and classification are confusing and difficult, and a matter for the specialist. A few comments only will be given relative to the more significant infections produced.

Favus is a disease which in man usually affects the scalp. The fungus (usually *Trichophyton schoenleinii*) apparently gains entrance through a hair follicle and grows between the outer cornified skin and the epithelial cells forming a yellowish mass with raised borders resembling a little shield (the scutulum). Considerable areas may be involved. The hairs themselves are also invaded. The central portion of the mycelium tends to die off, extending from the periphery. A similar organism (*Trichophyton quinckeanum*) producing mouse favus may produce disease in man as well.

The *ringworms* constitute a group of skin infections produced by various members of the genera *Trichophyton* and *Microsporum*. They may be transmitted from one person to another by contact, the spores of the fungi may be spread by fomites such as brushes, combs, and towels. They may in some cases be contracted from animals. It is possible that in some cases the fungi may live for a time saprophytically in stable litter. Ringworm may attack the hairy parts of the body or the smooth skin, occasionally the nails. In some types of ringworm of the smooth skin one finds a tendency for the fungus to spread from a center, forming a circle, the outer edge expanding and inflamed, the portion to the inside healing.

Athlete's foot is a dermatomycosis involving the folds of skin between the toes or on the sole of the foot, in which small, later coalescing, blisters are produced, the epithelium scales when dried, and redness and irritation persist. Apparently the disease is most often caused by *Trichophyton mentagrophytes* in one of its many forms.

THE DISEASE BLASTOMYCOSIS: THE GENUS BLASTOMYCES

The disease is sometimes termed Gilchrist's disease, from the individual who first described it in 1896. Later Gilchrist and Stokes were able to culture the causal fungus. The disease is not common, but is serious. In a large proportion of cases the initial lesions have been in the lungs. In other cases nodules were noted under the skin, but it is believed in all such cases the organisms had been carried by the blood stream. Abscesses may be formed in many tissues; the causal organism is *Blastomyces dermatitidis*.[2] In the lesions (nodules) produced in the body the cells are 3–24 μ in length, usually spherical, with a relatively thick wall. Multiplication may be by a process of budding somewhat like yeasts or the cell may elongate and form a septum. When grown on laboratory media a branching mycelium is developed. Conidia may be produced, sessile or on short conidiophores, spherical, oval, or pear-shaped, single-celled, and 2 μ by 3.5–5 μ.

Among other diseases of man produced by fungi are *sporotrichosis* caused by *Sporotrichum schenkii, chromoblastomycosis* caused by infection with *Phialophora verrucosa, mycetoma* (a deformed foot due to overgrowth of tissue as a result of an infection) caused by various species of actinomycetes and fungi.

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[2] The generic name *Blastomyces* has been a source of some confusion. The word means literally budding fungus, and as a common name has been used, particularly in Europe, to designate the pathogenic yeasts. The *Blastomyces dermatitidis* is not a yeast.

THE DISEASE ASPERGILLOSIS: THE GENUS ASPERGILLUS

The characteristics of this genus have already been described in Chapter 7. Aspergilli are important from two points of view. First, when present in considerable numbers upon moldy grain and other feed, they may produce a fatal type of pneumonia in birds of many kinds, particularly barnyard fowls. The species involved is usually *Aspergillus fumigatus*. The organism produces its mycelium within the lungs and air sacs and bears its grayish or greenish spores upon conidiophores within the air passages. A similar disease has been found to occur in man and animals, particularly the horse, when forced to breathe air containing the spores of this organism in great numbers. This and other species of *Aspergillus* are also important inasmuch as it seems very probable that they are capable of producing poisonous substances when growing in foods. There seems to be good evidence of the poisoning of animals due to the consumption of moldy corn, other grain, or silage with growth of this or related fungi. The nature of the poisonous principle is not known definitely.

PATHOGENIC YEASTS

Several species of yeasts or yeastlike fungi have been found to be pathogenic for man. Among these are *cryptococcosis* and thrush (*moniliasis*). The causal organisms do not produce ascospores, they belong in the group of anascosporogenous yeasts.

Cryptococcosis. This disease has been given several names; in fact, it may well serve as an illustration of desirability of stability in nomenclature. A convenient method of naming a disease is to append either *-osis* or *-iasis* to the name of the causal organism; the ending chosen is largely a matter of euphony. The organism causing this disease was recognized by Busse, who described it in 1895 as a yeast resembling morphologically the yeast of alcoholic fermentation, so he named the disease *saccharomycosis hominis* (of man). It was given the name *Saccharomyces neoformans* by Sanfelice. Then Vuillemin concluded that the organism was not a true yeast and placed it in the genus *Cryptococcus* as *C. hominis*, and so the disease because a *cryptococcosis*. Stoddard and Cutler (1916) mistakenly placed it in the fungus genus *Torula* as *T. histolytica*, and the name *torulosis* came into use for the disease. Inasmuch as the organism produced buds it was called in the European literature a blastomyces, hence the disease was a *blastomycosis*. This last name caused confusion with the blastomycosis, a distinct disease already considered caused by a member of the genus *Blastomyces.* Todd and Hermann (1936) suggested that the organism belongs in the genus *Debaryomyces,* which might well lead to another name for the disease. A confusion in terminology such as here indicated can eventually be settled only by international agreement. The name used in this discussion will be *cryptococcosis.*

The disease manifests itself in various ways. Perhaps most common (though relatively rare) in the United States it is a meningitis, while in Europe the disease is more usually characterized by skin lesions and by being more generalized. The disease is usually fatal.

In the tissues the organisms are spherical, producing buds in a typical yeastlike fashion, showing considerable variation in size and surrounded by a thick mucoid capsule. In culture the organism likewise has spherical cells, which multiply by budding. In young cultures the capsule is relatively thin, in older cultures it may be thick and somewhat asymmetrical. Diagnosis of the disease after isolation of the organism may be confirmed by intracerebral injection of mice in which a meningitis is produced fatal in one to two weeks.

Thrush is a relatively common and mild infection of mucous membranes. The causal organism (*Candida albicans*) has been given many different names. Diddens and Lodder (1942) list eighty-five of these that have appeared in the literature. One of the earlier workers (Zopf, 1890) incorrectly placed the organism in the fungus genus *Monilia;* from this the name *moniliasis* has commonly been given to the diseases caused by this yeast.

The infection manifests itself most frequently in infants still nursing. It is rare in well-nourished individuals and in those kept under reasonably good sanitary conditions. Usually the mucous membrane of the mouth is affected. The organism produces whitish areas or patches on the tonsils, gums, tongue, or cheeks, frequently forming a membrane which may be removed. This ability to produce a so-called false membrane sometimes leads to confusion with diphtheria. Occasionally the organism may invade moist skin, as in the axillae and between toes and fingers. Rarely the disease is generalized and proves fatal.

Examination of the membrane taken from the infected mucous membrane shows the organism producing a mycelium with accompanying typical budding yeast cells. The organism grows readily in culture media. On the surface of the medium it produces a typical yeast colony with budding yeast cells. Within the medium itself a false mycelium is produced, consisting of much elongated cells which bud off at the nodes smaller, typical yeast cells, which in turn bud to form a mass of spherical cells which function as spores and are designated as *blastospores.*

The organism is pathogenic for rabbits, injection producing abscesses in the heart muscle and kidneys, and sometimes lesions generally distributed through the body.

CHAPTER 46

Pathogenic Protozoa

The protozoa hold the same relative position among animals that bacteria and unicellular fungi such as the yeasts hold among plants. They pass their entire existence as single-celled individuals, although in some cases several or many cells may unite to form a colony. In no case, however, do the cells form a definite tissue or organ.

It has been noted in chapter 2 that there are many intergradations between the protozoa and bacteria. For example, the genus *Spirochaeta* is variously regarded. By some authors it has been grouped with the bacteria, by others with the protozoa, while others believe (and probably this view is justifiable) that it is a form intermediate between the two groups, partaking in part of protozoan and in part of bacterial characteristics. Our discussion of protozoa will be limited to three of the comparatively small number of genera and species which are known to produce disease in man. Many hundreds and even thousands of species are parasitic in the bodies of other animals. The genera to be considered are *Endamoeba, Trypanosoma,* and *Plasmodium*.

The Genus Endamoeba

The organisms of this genus belong to the class *Sarcodina* (*Rhizopoda*) of the protozoa. This group includes those protozoa which during their adult life move by means of protoplasmic projections from the cell termed *pseudopodia*. A definite fixed membrane is lacking, the protoplasm moving about by a kind of flowing motion. These protozoa reproduce by fission and by the formation of spores. Several hundred genera with numerous species have been described belonging to this group. One quite certainly, and probably two or three, are known to be pathogenic for man. *Endamoeba coli* is regarded as a more or less normal inhabitant of the intestinal tract in man, and somewhat similar species have been noted from the intestinal tracts of other animals.

It is possible to cultivate many species of amoebae upon artificial media. The amebae differ from organisms previously studied in that they utilize living cells as food. These cells are engulfed by the protoplasm which secretes digestive enzymes, and the food particles come to lie in a digestive vacuole or temporary stomach. If amebae are to be cultivated, they must be supplied with living cells for food. This may be accomplished for some kinds of amebae by growing bacteria upon the surface of a medium containing only sufficient nutrients for moderate development. Amebae can move about over the surface of this medium and engulf organisms as needed for food.

Endamoeba histolytica

Endamoeba histolytica is the specific cause of amebic dysentery in man. The organism may be found in stools by mixing a small quantity with physiological salt solution and placing upon a slide under a cover glass. The slide must be kept warm if motion is to be observed. The organism is usually actively motile, throwing out pseudopodia. The cell appears as a clear mass of protoplasm, sometimes tinged with green from the hemoglobin of the blood that has been ingested. Frequently the ameba contains red blood cells at various stages in digestion. It is not difficult to differentiate, in the larger cells at least, the two layers of protoplasm, the so-called endoplasm or inner portion, and the ectoplasm or outer layer. The ectoplasm is glasslike and refractive. The pseudopodia which are thrown out and by means of which the organism moves consist largely of ectoplasm. The endoplasm in active cells usually contains a considerable number of vacuoles. The ameba reproduces by binary fission. In the lumen of the intestine the cell tends to round up and become spherical and secrete about itself a wall and becomes a *cyst*. The nucleus divides to form four.

The cysts of *E. histolytica* fed to kittens produce a typical amebic dysentery with characteristic ulcerations of the intestinal wall. The cyst opens and the four nucleate amebae emerge. Several outbreaks have been reported in the United States. One particularly serious epidemic occurred in Chicago where improperly installed plumbing in two hotels made possible the contamination of the drinking water with sewage, with over fourteen hundred infections and nearly a hundred deaths.

The Genus Trypanosoma

The trypanosomes are parasites which occur in the blood of many species of animals, fish, reptiles, and birds, in some cases producing no apparent injurious effects, in other cases causing highly fatal diseases. The trypanosomal cell is elongated, usually more or less spindle-shaped, tapers to the ends, and is several times as long as broad. The anterior end is usually tipped with a flagellum, and longitudinally along one side of the cell is a thin, undulating membrane. The flagellum originates in the posterior portion of the cell near a deeply staining granule, extends along the outer edge of the membrane, and is free only at the very tip. The flagellum, therefore, is in part within the protoplasm of the cell, for a portion of its length attached to the edge of the undulating membrane, and usually free in part. The organism is actively motile. The individual cells can vary their shape to a considerable degree. Multiplication occurs through longitudinal splitting of the cell. It is known that some species of trypanosomes undergo a series of changes, or a developmental cycle, in the body of certain intermediate hosts.

Many species of trypanosomes may be cultivated in the laboratory. The method was first described in 1903 by Novy and MacNeal, who mixed equal portions of defibrinated rabbit blood and melted nutrient agar. This was slanted, allowed to solidify, and the trypanosomes inoculated into the water of condensation. By transferring the flagellates from one tube to another they successfully cultivated the organism for a considerable period of time.

Many species of trypanosomes, as has already been noted, appear in animals without seriously interfering with health. The rat frequently harbors trypanosomes in the blood. Birds, fish, and reptiles often have in their blood trypanosomes or representatives of closely related genera. Many diseases of animals, such as surra in horses and cattle, the nagana or tsetse fly disease in horses, cattle, and camels, and a considerable list of similar diseases affecting domestic and wild animals, have been described as due to infection with pathogenic trypanosomes.

Trypanosoma gambiense

Trypanosoma gambiense (with *T. rhodesiense*) is the cause of human trypanosomiasis or sleeping sickness. The disease has been known among the negroes on the western coast of Africa for more than a century. Within recent years it has spread to a marked degree, and some districts of central Africa have been completely depopulated and rendered uninhabitable. It is the principal foe to the development and settlement of large areas in that country. At the present time it is spreading slowly, but vigorous methods are being taken to control it.

Morphology. The organism is 1.4–2 μ by 17–28 μ. The free flagellum may be from one-fourth to one-third the length of the body. The undulating membrane is relatively narrow. The organism is actively motile when examined in fresh blood.

Disease Production. The disease may be produced by the injection of infected blood into the monkey, dogs, cats, and various other laboratory animals. The disease in man is insidious in its onset. At first the organism appears in the blood, there usually is more or less irregular fever, and the lymph glands become swollen. In the second stage pains develop in the back. The patient becomes exceedingly drowsy and can be aroused only with difficulty. Finally he settles into a state of coma and dies. The organism may be demonstrated in this second stage in the cerebrospinal fluid. The disease is usually fatal in untreated cases, but may run a somewhat chronic course. No method of immunization has been developed, but specific drugs have been developed which have proved quite successful in curing the disease.

Transmission. One of the bloodsucking flies, the so-called tsetse fly (*Glossina palpalis*), transmits the parasite. The fly after it has fed upon the blood of a patient having the disease may infect laboratory animals within the next two days, when allowed to feed upon them. It then loses its power of infection, but regains it in about three weeks. It seems probable therefore that the organism passes through certain developmental stages within the body of the fly, and after these changes the insect again becomes infective. Some effort is being made to control the disease by destruction of the breeding places of these flies along water courses and to prevent these insects from biting infected individuals.

The Genus Plasmodium

Three (possibly four) species of protozoa, commonly included in the genus *Plasmodium*, have been determined to be the specific causes of three types of malaria in man. In every case the organism goes through certain changes (a developmental cycle) in the red blood cells of man, and the remainder of the life cycle is carried out in the digestive tract and tissues of the mosquito. Plasmodium was first described by Laveran in 1880, and since that time the three types have been differentiated.

Plasmodium vivax

Plasmodium vivax is the cause of the disease of man known as tertian malaria, the common malaria or "ague" of temperate climates.

Morphology and Life History. The organism when it first enters the human blood is small and ameboid. It soon penetrates a red blood cell, enlarges, and develops in the interior of the cell until this is filled. It then segments to form a rosette of small merozoites. These escape from the red blood corpuscle, each one attaches itself to another cell, penetrates it, and begins development anew. This growth to full size followed by merogony and infection of new cells may be repeated several times. This constitutes the asexual phase in the life history of the organism. The disease is always characterized by chills and fever which develop at the time when the organisms escape from the corpuscles, and it is possible that certain poisonous materials are liberated at this time. In order to complete the life cycle, the organism must pass into the body of a mosquito. This insect takes it up with blood. In the gut of the mosquito two types of gametes are formed, corresponding to eggs and sperms. Each egg is fertilized by a sperm and burrows into the stomach wall. Here it becomes considerably enlarged and breaks up into a number of cells called sporoblasts. The contents of these in turn undergo multiplication to form large numbers of delicate filamentous cells termed sporozoites. The oocyst wall ruptures, and the sporozoites pass into the body cavity of the mosquito. From here they make their way to the salivary glands and pass into the blood of the individual next bitten by the insect. It is evident that during the sexual cycle of the organism, the insect remains non-infective. From eight to ten days (or longer, depending upon temperature) usually elapse between the time the blood is taken into the body of the mosquito and the appearance of the organism in the poison glands. The malaria produced by *Plasmodium vivax* is usually benign, rarely terminating fatally. The chills and fever appear every two days, as this is the time required for the organism to pass through its asexual cycle in the human blood.

No method of immunization has been developed. Treatment is by use of specific drugs, including quinine and atabrin. Malaria, as was emphasized above, is transmitted to man only by the bite of an infected mosquito. Prevention of the disease necessitates the destruction of the breeding places of mosquitoes, and when such bodies of water cannot be drained, they must be oiled to kill the larvae.[1] Care must likewise be used to see that the mosquito does not bite malarial patients and be thus rendered infective. Lastly mosquitoes should be prevented from biting healthy individuals as far as possible.

Plasmodium malariae

Plasmodium malariae produces the so-called quartan malaria in which the interval between the paroxysms of fever is seventy-two hours. The disease is relatively benign and is transmitted in the same manner as the preceding.

Plasmodium falciparum

The malarias produced by *Plasmodium falciparum* are malignant and do not yield readily to quinine treatment. Two types are known, a quotidian in which the asexual cycle is complete in twenty-four hours and a tertian in which it is complete in forty-eight hours. These are transmitted in the same manner as are the preceding types. They are the most usual malarias of the tropics.

Plasmodium ovale

Plasmodium ovale, a rather rare form, resembles *P. malariae* in its early stages but the sporulating forms have only eight merozoites. It is chiefly characterized by the fact that the parasitized blood cells are oval in shape and drawn nearly to a point at one end. Only in recent years has the validity of this species been recognized.

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[1] The mosquito passes the first stages of its life in the water; only the mature insect is a bloodsucker.

APPENDICES

APPENDIX A

Keys to Orders, Families, and Genera of Bacteria

Chapter 4 discusses the general problems of classification and nomenclature of the bacteria, but the list of genera there described is not inclusive. The succeeding pages constitute an effort to give a reasonably complete list of the bacterial genera for reference and to assist students who are attempting to identify cultures.

It should be appreciated that there is yet much diversity of opinion relative to the naming and classification of bacteria and that the following keys and descriptions are necessarily subject to modification and revision.

In general the names used are those of Bergey's *Manual* (6th ed.). They have been modified in some cases to conform to later Opinions of the International Judicial Commission on Bacteriological Nomenclature.

Key to the Orders and Suborders of Bacteria (Schizomycetes)

a. Not obligate intracellular parasites.
b. Motile or non-motile. When motile possessing flagella. Cells not flexuous.
c. No development into filaments, no formation of mycelium made up of branched hyphae. Order *Eubacteriales*
d. No photosynthetic chlorophyll-like pigments.
e. Not attached to substrate by a stalk. Suborder *Eubacteriineae*
ee. Attached to substrate by stalk or globule of mucus. Suborder *Caulobacteriineae*
2d. With photosynthetic green pigments. Frequently contain sulfur. Often with red or purple pigments. Suborder *Rhodobacteriineae*
2c. Either elongate cells in chains or developing as branched hyphae into a mycelium.
d. Forming a mass of branched hyphae (mycelium), which may produce conidia by segmentation. Order *Actinomycetales*
2d. Forming chains of cells frequently enclosed in a firm gelatinous sheath or tube. Order *Chlamydobacteriales*
2b. Motile, either by swimming by cell flexion or by creeping on the substrate.

c. Motility, swimming by cell flexion of spiral cells. Order *Spirochaetales*

2c. Move by creeping along surface of substrate. Order *Myxobacterales*

2a. Obligate intracellular parasites. Order *Rickettsiales*

Key to the Families of the Eubacteriineae, the Typical or True Bacteria

- a. Cells require organic media for growth, not autotrophic.
  - b. Straight rods producing endospores. 1. *Bacillaceae*
  - bb. Not endosporous straight rods.
    - c. Straight or curved rods with true polar flagella. 2. *Pseudomonadaceae*
    - cc. Rods or cocci, if motile, flagella not polar.
      - d. Free-living soil rods, capable of fixing atmospheric nitrogen. 3. *Azotobacteraceae*[1]
      - dd. Not nitrogen-fixing free-living soil rods.
        - e. Not growing in symbiosis with root of leguminous plant.
          - f. Cells spherical but never in chains.
            - g. In irregular masses, not typically in pairs, not anaerobes. Gram-positive or gram-negative. 4. *Micrococcaceae*
            - gg. Typically in pairs if aerobic, in masses if anaerobic. Gram-negative. 5. *Neisseriaceae*
          - ff. Cells rod-shaped or spherical; if the latter, typically in chains.
            - g. Gram-positive cocci or rods.
              - h. Cocci in chains or rods. Strongly fermenting sugars with production of acids. Nitrates not reduced. Microaerophilic to anaerobic. 6. *Lactobacteriaceae*[2]
              - hh. Rods, usually aerobic, sometimes microaerophilic. Less active acid producers. Nitrates may or may not be reduced. 7. *Corynebacteriaceae*
            - gg. Gram-negative rods.
              - h. Cells not showing marked bipolar staining, not requiring special growth accessory substances which are not found in peptone. 8. *Enterobacteriaceae* 9. *Achromobacteraceae*
              - hh. Cells either showing marked bipolar staining, particularly in animal tissues, or requiring in addition to peptone certain growth promoting substances as found in blood or in certain plant tissues. 10. *Parvobacteriaceae*
        - ee. Growing symbiotically with the roots of leguminous plants. 11. *Rhizobiaceae*
- aa. Cells do not require organic media for growth, either obligately or facultatively autotrophic. 12. *Nitrobacteraceae*[3]

Key to the Genera of the Family Bacillaceae

- a. Cells aerobic, producing the enzyme catalase. 1. *Bacillus*
- aa. Cells anaerobic or microaerophilic; catalase not known to be produced. 2. *Clostridium*

[1] Derived from *Azotobacter*, hence spelling *Azotobacteraceae*, not *Azotobacteriaceae*.

[2] The legitimacy of the family name *Lactobacteriaceae* may be questioned; there is no genus name *Lactobacterium*.

[3] Name derived from *Nitrobacter*, hence family name *Nitrobacteraceae* and not *Nitrobacteriaceae*.

Key to the Genera of the Family Pseudomonadaceae[4]

a. Cells not bent, straight rods.
  b. Oxidize ethyl alcohol to acetic acid, the vinegar bacteria. 1. *Acetobacter*
  bb. Do not oxidize ethyl alcohol to acetic acid.
    c. Yellow pigment, no water soluble pigment. 2. *Xanthomonas*
    cc. Pigment if present water soluble. 3. *Pseudomonas*
aa. Cells more or less bent, or spirally curved.
  b. Cells relatively short, and motile by means of a single (sometimes two or three) polar flagellum. 4. *Vibrio*
  bb. Cells relatively longer and motile by a tuft of polar flagella. 5. *Spirillum*

Key to the Genera of the Family Micrococcaceae

a. Cells are not arranged in regular cubes, or tetrads, usually separate or in irregular masses. 1. *Micrococcus*
aa. Cells in tetrads or cubes.
  b. Cells in animal body or in special media in regular tetrads. 2. *Gaffkya*
  bb. Cells occur regularly in packets or cubes. 3. *Sarcina*

Key to the Genera of the Family Neisseriaceae

a. Cells usually in pairs, with proximal sides flattened. Usually 1 μ in diameter. Aerobes or anaerobes. 1. *Neisseria*
aa. Cells usually in masses, rarely paired. Obligate anaerobes. Cells usually about 0.5 μ in diameter. 2. *Veillonella*

Key to the Tribes of the Lactobacteriaceae (Lactobacillaceae)

a. Cells spherical, frequently in pairs or in chains. *Streptococceae*
aa. Cells rod-shaped, frequently in chains. *Lactobacilleae*

Key to the Genera of the Tribe Streptococceae

a. Cells usually in pairs. Often parasitic. *Diplococcus*
aa. Cells typically in chains.
  b. Ferment glucose to lactic acid, little or no production of CO_2. Many parasitic. *Streptococcus*
  bb. Ferment glucose with production of a mixture of acids and CO_2. All saprophytic. *Leuconostoc*

Key to the Genera of the Tribe Lactobacilleae

a. Carbohydrates fermented with production of lactic acid primarily.
  b. Microaerophilic. Catalase negative. *Lactobacillus*
  bb. Aerobic. Catalase positive. *Microbacterium*
aa. Carbohydrates fermented with production of compounds other than lactic acid, or in addition to lactic acid, with CO_2.
  b. Carbohydrates fermented with production of propionic and acetic acids Catalase positive. *Propionibacterium*

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[4] In addition there are listed in Bergey's *Manual* (6th ed.) the following genera of relatively little economic significance: *Methanomonas*, *Protaminobacter*, *Mycoplana*, *Desulfovibrio*, *Cellvibrio*, *Cellfalcicula*, and *Thiospira*.

bb. Carbohydrates fermented with production or butyric and acetic acid. Usually catalase negative. *Butyribacterium*

Key to the Genera of the Family Corynebacteriaceae

a. Non-motile rods.
  b. Catalase positive. *Corynebacterium*
  bb. Catalase negative. *Erysipelothrix*
aa. Motile rods. *Listeria*

Key to the Tribes of the Family Enterobacteriaceae[5]

a. Non-chromogenic. Do not produce a red or orange pigment.
  b. Lactose promptly fermented with formation of acid or of acid and gas.
    c. Produce acid and gas from lactose. Not plant parasites. 1. *Escherichieae*[6]
    cc. Produce acid or acid and gas from lactose. Plant parasites. 2. *Erwinieae*
  bb. Lactose not fermented promptly.
    c. Urea decomposed promptly. 3. *Proteae*
    cc. Urea not decomposed promptly. 4. *Salmonelleae*
aa. Chromogenic, producing a pink, red, or orange pigment. 5. *Serratieae*

Key to the Genera of the Tribe Escherichieae

a. Acetyl methyl carbinol not produced, i.e., Voges-Proskauer test negative. Methyl red test positive. 1. *Escherichia*
aa. Acetyl methyl carbinol produced, i.e., Voges-Proskauer test positive. Methyl red test negative. 2. *Aerobacter*

(The generic name *Klebsiella* is sometimes used to designate the mucoid or capsulated forms isolated from the body as from respiratory infections. *Paracolobactrum* is sometimes applied to species that ferment lactose slowly.)

The Tribe Erwinieae

One genus only, *Erwinia.*

The Tribe Proteae

One genus only, *Proteus.*

Key to the Genera of the Tribe Salmonelleae

a. Ferment glucose with acid and gas; rarely gas absent. *Salmonella*
aa. Ferment glucose with acid only. *Shigella*

The Tribe Serratieae

One genus only, *Serratia.*

Key to the Genera of the Family Achromobacteraceae

a. Not producing yellow or orange pigment.
  b. No acid from carbohydrates. Milk turned alkaline. *Alcaligenes*
  bb. Some acid from carbohydrates. Milk rarely alkaline. *Achromobacter*
aa. Producing yellow or orange pigment. *Flavobacterium*

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[5] This family name will probably be replaced by *Bacteriaceae.*

[6] This tribal name will probably be replaced by *Bacterieae.*

Key to the Tribes of the Family Parvobacteriaceae

a. Growth not dependent upon presence of growth-promoting factors of blood or of plant tissues in media.
b. Aerobic, facultative, or microaerophilic.
c. Bipolar staining evident. Usually acid from carbohydrates. 1. *Pasteurelleae*
cc. No bipolar staining. No acid from carbohydrates. 2. *Brucelleae*
bb. Anaerobic. 3. *Bacteroideae*
aa. At least when first isolated, growth dependent upon presence of growth factors from serum or plant tissues. 4. *Hemophileae*

Key to the Genera of the Tribe Pasteurelleae

a. Producing honey-like growth on potato. Cells sometimes becoming elongate and with tendency to branching. *Malleomyces*
aa. Not as a.
b. Rods relatively short, showing bipolar staining. *Pasteurella*
bb. Pleomorphic, short to relatively long rods. *Actinobacillus*

The Tribe Brucelleae

A single genus, *Brucella*.

Key to the Genera of the Tribe Bacteroideae

a. Cells with rounded ends, not pointed or tapering. 1. *Bacteroides*
aa. Cells with pointed or tapering ends. 2. *Fusobacterium*

Key to Genera of Tribe Hemophileae

a. Cells non-motile.
b. Cells aerobic or facultative.
c. Cells occurring singly. 1. *Hemophilus*
cc. Cells usually in pairs. 2. *Moraxella*
bb. Cells obligately anaerobic. 3. *Dialister*
aa. Cells motile. 4. *Noguchia*

Key to the Genera of the Family Rhizobiaceae

a. Violet pigment not produced when grown on artificial media.
b. Grow symbiotically in nodules of roots of legumes, fixing nitrogen. 1. *Rhizobium*
bb. Not growing as b. Usually producing galls or root hypertrophies. 2. *Agrobacterium*
aa. Violet pigment produced when grown on artificial media. 3. *Chromobacterium*

Key to Genera of the Family Nitrobacteraceae

a. Organisms oxidize ammonia or nitrite.
b. Organisms oxidize ammonia to nitrite.
c. Cells not embedded in gelatinous masses.
d. Cells spherical. *Nitrosococcus*
dd. Cells elongate.
e. Cells not spiral. *Nitrosomonas*
ee. Cells spiral. *Nitrospira*

cc. Cells embedded in gelatinous masses, forming a zoogloea.
d. Membrane about zoogloeal mass. *Nitrosocystis*
dd. No membrane about the zoogloeal mass. *Nitrosogloea*
bb. Organisms oxidize nitrite to nitrate.
c. No zoogloeal mass. *Nitrobacter*
cc. In zoogloeal masses. *Nitrocystis*
aa. Organisms do not oxidize ammonia or nitrites.
b. Oxidize elementary hydrogen. *Hydrogenomonas*
bb. Oxidize sulfur and thiosulfates. *Thiobacillus*

Key to the Families of the Order Actinomycetales

a. Cells short to long, rarely branching, with no development of a much-branched mycelium. All species produce acid-fast cells under the right environmental conditions. 1. *Mycobacteriaceae*
aa. Cells elongate, producing a branched mycelium.
b. Mycelium breaks up into rod-shaped or even spherical segments which function as spores. 2. *Actinomycetaceae*
bb. Mycelium does not break up as under a, but special branches pinch off spores (conidia) at the tip. 3. *Streptomycetaceae*

Genera of the Family Mycobacteriaceae

One genus only, *Mycobacterium.*

Key to the Genera of the Family Actinomycetaceae

a. Obligate aerobes. Occasionally some development of aerial mycelium, some species partially acid-fast. 1. *Nocardia*
aa. Anaerobes or microaerophiles, nonacid-fast. 2. *Actinomyces*

Key to the Genera of the Family Streptomycetaceae

a. Conidia produced in chains from aerial hyphae. 1. *Streptomyces*
aa. Conidia produced singly from tips of aerial conidiophores. 2. *Micromonospora*

Key to the Families of the Order Spirochaetales

a. Relatively long spirals, 30–500 μ. Free-living or parasitic in certain shellfish (mussels). *Spirochaetaceae*
aa. Relatively more slender and shorter, usually 4–16 μ in length. Generally parasitic, frequently pathogenic. *Treponemataceae*

Key to the Genera of the Family Spirochaetaceae

a. No obvious periplast membrane present. *Spirochaeta*
aa. Periplast membrane or crest present.
b. Free-living in marine ooze. *Saprospira*
bb. Parasitic in molluscs (crystalline style). *Cristispira*

Key to the Genera of the Family Treponemataceae

a. Readily stained with usual aniline dyes. *Borrelia*
aa. Stain with difficulty with usual dyes, readily with Giemsa.
b. Strictly anaerobic. *Treponema*
bb. Aerobic. *Leptospira*

Key to the Families of the Order Chlamydobacteriales

- a. Cells of filaments do not contain sulfur granules.
  - b. Filaments free in water, not attached by basal holdfast. *Chlamydobacteriaceae*
  - bb. Filaments differentiated into a holdfast base and a tip. *Crenotrichaceae*
- aa. Cells of filaments contain sulfur granules when growing in sulfur waters. *Beggiatoaceae*

Key to Genera of Family Chlamydobacteriaceae

- a. Sheaths not impregnated with iron. *Sphaerotilus*
- aa. Sheaths impregnated with iron.
  - b. Filaments showing false branching. *Clonothrix*
  - bb. False branching absent or rare. *Leptothrix*

Genera of the Family Crenotrichaceae

One genus only, *Crenothrix.*

Key to the Genera of the Family Beggiatoaceae

- a. Filaments attached by holdfast at base. Not motile. *Thiothrix*
- aa. Filaments free, move by a gliding motion.
  - b. Occur singly, not united by slime.
    - c. Filaments not permanently coiled. *Beggiatoa*
    - cc. Filaments coiled or spiral. *Thiospirellopsis*
  - bb. In bundles in common slime sheath. *Thioplica*

Key to the Families of the Order Rickettsiales

- a. Parasitic in tissue cells, do not occur in or on red blood cells.
  - b. Found in arthropods, some transmitted to vertebrates through arthropod vectors. *Rickettsiaceae*
  - bb. Found in vertebrate tissues, not transmitted by arthropods. *Chlamydozoaceae*
- aa. Parasitic in or on red blood cells of vertebrates. *Bartonellaceae*

APPENDIX B

Keys to the Families and Genera of the Yeasts and to the Species of the Genus *Saccharomyces*

The classification of this group has been largely drawn from the work of the Baarn and Delft schools in Holland, particularly from the monographs of Stelling-Dekker, Lodder, and Diddens and Lodder. There has been rapid development in our knowledge of this group so that any classification must be accepted as quite tentative.

Key to the Families and Subfamilies of Yeasts

a. Asci and ascospores produced. *Saccharomycetaceae*[1]
2a. No asci or ascospores produced.
  b. Spores not borne on basidia.
    c. No conidia produced.
      d. Cells contain a pink or red carotenoid pigment. *Rhodotorulaceae*
      2d. Cells usually without pink or red pigment, if present not carotenoid. *Torulopsidaceae* or *Cryptococcaceae*
        e. Without a pseudomycelium, or such imperfectly developed. Without any specialized spore-producing apparatus. *Torulopsidoideae* or *Cryptococcoideae*
        2e. With a pseudomycelium and a specialized spore-producing apparatus. *Mycotoruloideae* or *Candidoideae*
    2c. Conidia produced. *Nectaromycetaceae*
  2b. Spores produced on a basidium. *Sporobolomycetaceae*

Key to the Genera of the Family Saccharomycetaceae

a. Ascospores spherical or relatively short, never needle-shaped or elongate spindle-shaped. Not primarily plant or animal parasites. (Except *Debaryomyces.*)
  b. Vegetative cells not forming a true mycelium with cross walls, sometimes pseudomycelium of united budding cells.
    c. Vegetative reproduction by budding, not by transverse fission of the cell.
      d. Vegetative reproduction by multilateral, not by bipolar, budding.
        e. Not producing a dull dry pellicle promptly in wort.
          f. Ascospores smooth, not warty.

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[1] The family name *Endomycetaceae* is sometimes used in place of *Saccharomycetaceae.* The latter name has priority and should be used.

g. Vegetative cells spherical, ovoid to elongate. Ascospores spherical, oval or occasionally kidney-shaped, one to four in ascus.

h. No conjugation immediately preceding ascus formation. ***Saccharomyces***

2h. Conjugation, either isogamous or heterogamous before ascus formation. ***Zygosaccharomyces***

2g. Vegetative cells spherical. Ascospores spherical, each with central oil globule; one, sometimes two in ascus. Abortive conjugation tubes, but no union. ***Torulaspora***

2f. Ascospores warty, spherical, with oil drop. Ascus usually one-spored.

g. Vegetative cells relatively small, usually spherical. Hetero- or isocopulation. No ridge on spores. *Debaryomyces*

2g. Vegetative cells relatively large, cells oval. No copulation, but with abortive conjugation tubes. Spores with ridge or border. Asci retort-shaped. ***Schwanniomyces***

2e. Producing a dull dry pellicle promptly on wort.

f. Nitrates are assimilated. Splits aesculin. *Hansenula*

g. Asci from diploid cell. subg. ***Hansenula***

2g. Asci follow iso- or heterocopulation. subg. *Zygohansenula*

2f. Nitrates assimilated. Do not split aesculin.

g. Ascospores smooth, round, angular or hat-shaped; cells in young cultures long-oval to elongate. ***Pichia***

h. Asci apparently from diploid cells, but uncertain. No conjugation. subg. ***Pichia***

2h. Asci produced following copulation. subg. *Zygopichia*

2g. Ascospores warty, round; cells in young cultures spherical to short oval. ***Debaryomyces***

2d. Vegetative reproduction by bipolar rather than by multilateral budding.

e. Heterocopulation of mother cell and bud, second bud forms, becoming ascus. Ascospores warty. ***Nadsonia***

2e. Not as e. Ascospores smooth.

f. Cells relatively small. No copulation of ascospores before germination. Cells lemon-shaped or long ovals. Ascospores spherical to hat-shaped. Two to four per ascus. ***Saccharomycodes***

2f. Cells relatively larger, sausage or lemon-shaped. Spores spherical, four per acus. Ascospores copulate before germination. ***Hanseniaspora***

2c. Vegetative reproduction by transverse fission of the cell, not by budding. ***Schizosaccharomyces***

2b. Vegetative cells forming a true mycelium with cross walls.

c. Mycelium may break into arthrospores (oidia). Asci develop in branched mycelium. No budding cells or blastospores (conidia). ***Endomyces***

2c. Mycelium produced, sometimes arthrospores (oidia), and always blastospores (conidia) and budding cells. Asci either in hyphae or separate. Isogamic copulation. ***Endomycopsis***

2a. Ascospores elongate, slender, needle or spindle-shaped. Parasitic.

b. Ascospores needle-shaped, ascus one-spored. Not cultivated (Parasitic in a water flea.) ***Monosporella***

2b. Ascospores long spindle-shaped, ascus eight-spored.

c. Ascospores with long filament (flagellum-like) at one end. Ascospores in two bundles, one at each end of ascus. Cultivated, producing fermentation. Plant parasites. *Nematospora*

2c. Ascospores without terminal filament. Not cultivated. Parasitic in alimentary tract of an insect. *Coccidiascus*

Key to Species of Saccharomyces

(Adapted from Dekker)

All species produce alcohol and CO_2 from the three monosaccharides, dextrose, levulose, and mannose. For species separation six other sugars are used, the monosaccharide galactose, the disaccharides sucrose, maltose, lactase, and melibiose, and the trisaccharide raffinose.

a. Galactose +

b. Sucrose —

c. In wort promptly relatively long chains 1. *Saccharomyces dairensis*

2c. In wort cells single or in pairs.

d. Ascus one-spored. 2. *S. unisporus*

2d. Ascus more than one-spored. 3. *S. globosus*

2b. Sucrose +

c. Lactose —

d. Maltose —

e. Young cells in wort small (3.5–5.5 μ by 4–7 μ), spherical to oval. 4. *S. exiguus*

2e. Young cells in wort larger (3.5–5 μ by 5–10 μ), oval. 5. *S. manginii*

2d. Maltose +

e. Raffinose — 6. *S. chodatii*

2e. Raffinose +

f. Melibiose —, Raffinose ⅓

g. Old cells on wort agar, very long, to 30 μ. Maltose poorly fermented. Young cells in wort 3–5 μ by 7–10 μ, elliptical-elongate. 7. *S. tubiformis*

2g. Old cells on wort not as in g. Maltose strongly fermented.

h. Old cells on wort agar very large and irregular, as though bud separation incomplete, producing irregular "cell-complexes," in broth, young cells 3–6 μ by 4.5–9 μ, older cells elongate. 8. *S. paradoxus*

2h. Not as h.

i. Cells elongate on wort agar 2–4.5 μ by 6–14 μ. Young cells in wort 3–5.5 μ by 5–9 μ. 9. *S. odessa*

2i. Not as i

j. In wort young cells relatively large, 4–8 μ by 9–18 μ, short to long oval. 10. *S. willianus*

2j. In wort cells relatively more slender or smaller.

k. In wort young cells 1–2 times as long as broad, 3–7 μ by 5–14 μ. Cells spherical, oval, ellipsoidal, or pear-shaped. 11. *S. cerevisiae*

2k. In wort young cells 3–4 times as long as broad. 2.5–4 μ by 9–11 μ. Oval to cylindrical. 12. *S. intermedius*

f. Melibiose + Raffinose complete.

g. In wort young cells in long bud unions of long-oval cells. 13. *S. pastorianus*

2g. Young cells in wort single or in pairs or threes.

h. Young cells in wort very elongate, 4–6 times as long as broad. 14. *S. validus*

2h. Young cells in wort not 4–6 times as long as broad.

i. Young cells in wort short-oval, 3–5 μ by 7–10 μ. 15. *S. carlsbergensis*

2i. Young cells in wort larger.

j. Young cells in wort long-oval, 4–5.5 μ by 9–13 μ. 16. *S. logos*

2j. Young cells in wort oval and elongate, 3–6 μ by 7–15 μ, on wort agar filamentous, 2–5 μ by 4.5–20 μ. 17. *S. uvarum*

2c. Lactose + 18. *S. fragilis*

2a. Galactose —

b. Maltose —

c. Melibiose —, Raffinose ⅓ fermented.

d. Cells elongate, in chains. 19. *S. muciparis*

2d. Cells spherical or oval. 20. *S. chevalieri*

2c. Melibiose + Raffinose completely fermented. 21. *S. microellipsoides*

2b. Maltose +

c. Raffinose — 22. *S. heterogenicus*

2c. Raffinose +

d. Cells oval. 23. *S. oviformis*

2d. Cells long-oval to elongate. 24. *S. bayanus*

Key to the Genera of the Torulopsoideae (Cryptococcoideae)

(Adapted from Lodder)

a. Cells predominantly lemon-shaped, bipolar budding. *Kloeckera*

2a. Cells not lemon-shaped.

b. Cells predominantly triangular, with budding at the three corners. *Trigonopsis*

2b. Cells not triangular.

c. Cells predominantly flask-shaped, bud usually from a single cell with a broad base. *Pityrosporum*

2c. Cells predominantly spherical, oval, or cylindrical.

d. No pellicle on wort, or after a long time a moist, somewhat slimy pellicle.

e. On Gorodkowa agar characteristic short buds which much resemble the copulation outgrowths of *Zygosaccharomyces*. *Asporomyces*

2e. No formation of such outgrowths. *Torulopsis*

2d. A mat-like dry pellicle forms immediately on wort.

e. Cells usually cylindric. Multiplication through budding (knospung), the bud is not separated by fission from the mother cell. *Mycoderma*

2e. Cells polymorphic. Multiplication by budding (sprossung), the bud is often separated through fission from the mother cell. *Schizoblastosporion*

Key to the Genera of the Mycotoruloideae (or Candidoideae)

(Adapted from Diddens and Lodder)

a. Blastospores but no arthrospores developed.
  b. Cells often arch-pointed; under aerobic conditions strong acid formation. Cells die quickly. *Brettanomyces*
  2b. Cells not arch-pointed; under aerobic conditions no strong acid formation. *Candida*
2a. Both blastospores and arthrospores developed. *Trichosporon*

APPENDIX C

Key to Families and Genera of Certain Common Molds

The following illustrated key should enable the student to determine the genus (generic name) of most (not all) of the molds commonly encountered in bacteriological work and growing in bacteriological media. If an organism is not found described in the list here given, some more complete compendium should be used.[1] About a hundred and forty genera of molds have been described as found in soils; of these genera about forty-five are given below. Genera not included are observed quite rarely.

a. Vegetative mycelium with no or relatively few crosswalls or septa. Sexual spores produce resting spores or zygospores.

b. Sporangia always produced, in a few forms conidia may also be present.

c. Sporangium containing a columella; zygospores with none or at most very few appendages.

d. Wall of sporangium without thickening or cuticularization, usually easily broken.

e. Sporangia of one type only.

Family I. *Mucoraceae* (p. 642)

2e. Sporangia of two sorts, primary and large, and secondary and small.

Family II. *Thamnidiaceae* (p. 642)

2d. Upper portion of sporangium thickened and cuticularized, lower portion thin and easily ruptured.

Family III. *Pilobolaceae* (p. 642)

2c. Columellae not present in sporangium; zygospores with a tight covering of hyphae.

Family IV. *Mortierellaceae* (p. 642)

2b. Sporangia rare, conidia always produced. Conidia solitary, sometimes small sporangia on special bladder-like fertile hyphae.

Family V. *Choanephoraceae* (p. 642)

2a. Vegetative mycelium with numerous crosswalls or septa. Spores (conidia) not borne in a spore-case or sporangium, not in asci, borne on conidiophores of various types.

The *Fungi Imperfecti*

---

[1] One of the most convenient keys with descriptions of molds which may be encountered in the bacteriological laboratory is Gilman's *A Manual of the Soil Fungi*, 1945.

- b. Conidiophores separated, not united into columns or stalks or globose bodies.
  - c. Neither hyphae nor conidia dark or smoky in color; conidia usually clear, sometimes bright-colored.
    Family VI. *Moniliaceae* (p. 644)
  - 2c. Either hyphae or conidia dark or smoky in color, usually both.
    Family VII. *Dematiaceae* (p. 648)
- 2b. Conidiophores united into columns or stalks, or into globose, compact bodies.
  - c. Conidiophores closely united as parallel strands into a cylindric column or stalk (called a synnema or a coremium).
    Family VIII. *Stilbaceae* (p. 652)
  - 2c. Conidiophores closely united to form a more or less spherical, unstalked body (a sporodochium).
    Family IX. *Tuberculariaceae* (p. 654)

Key to the Genera of the Family Mucoraceae

a. Producing stolons (runners) which form holdfasts (rhizoids) on contact with substrate.

b. Sporangiophores not branched.

c. Sporangiophores borne at nodes of stolons. Sporangia spherical. *Rhizopus.*
(Fig. A-1. (1) sporangiophores, sporangia; (2) rhizoids; (3) stolon; (4) spores; (5) columella and apophysis.)

2c. Sporangiophores borne on internodes of stolons. Sporangia pear-shaped. *Absidia.*
(Fig. A-2. (1) sporangiophores, sporangia; (2) rhizoids; (3) stolon; (4) spores; (5) sporangium, columella. Adapted from van Tieghem.)

2b. Sporangiophores branched, large terminal and small lateral sporangia usually in whorls. *Actinomucor*
(Fig. A-3. (1) large terminal and small lateral sporangia; (b) columella of terminal sporangium with lateral sporangia. Adapted from Lindner.)

2a. Not producing stolons or holdfasts.

b. Sporangiophores long, with a metallic sheen, never branched. *Phycomyces.*
(Fig. A-4. Sporangium, columella, spores.)

2b. Sporangiophores branched or unbranched, not as b.

c. Sporangiophores with strongly curved lateral branches. *Circinella.*
(Fig. A-5. Sporangiophores, sporangia and columellae. Adapted from Lindner.)

2c. Sporangiophores branched or unbranched, if the former, not with strongly curved lateral branches.

d. Zygospores rare or absent, not produced on special hyphae, gametes equal. *Mucor.*
(Fig. A-6. (1) sporangium, sporangiophore, columella: (2) spores (3, 4) branches: (5) simple sporangiophores: (6) chlamydospores: (7) zygospore.)

2d. Zygospores relatively abundant, on special branched hyphae, gametes unequal. *Zygorhynchus.*
(Fig. A-7. (1) sporangium, columella, sporangiophore: (2) branched sporangiophore: (3) spores: (4) zygospore.)

Family Thamnidiaceae

One genus. *Thamnidium.*
(Fig. A-8. (1) branched sporangiophore with primary and secondary sporangia; (2) sporangioles; (3) spores; sporangium with columella; (5) zygospore.)

Key to Genera of Family Pilobolaceae

a. Sporangium with upper portion only of sporangium thick and cuticularized, lower part swollen, thick-walled and diffluent. *Pilaira.*
(Fig. A-9. (1) spores; (2) sporangiophore; (3) sporangium. Adapted from van Tieghem.)

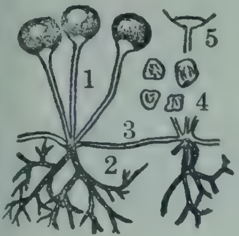
2a. Sporangium wholly thick-walled and glutinous; sporangiophore much swollen below the sporangium, breaking below sporangium at maturity and projecting the sporangium. *Pilobolus.*
(Fig. A 10. (1) sporangium and sporangiophore; (2) portion bulbous mycelium. After Zopf.)

Family Mortierellaceae

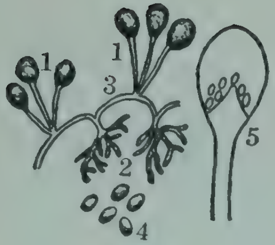
One genus. Sporangium without columella, terminal. Sporangiophores frequently branched. *Mortierella.*
(Fig. A-11. (1) branched sporangiophore with sporangia. Adapted from van Tieghem.)

Family Choanephoraceae

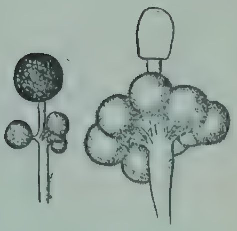
One genus. Sporangia rare, conidiophores swollen at tip, with papillae each bearing a conidium. *Cunninghamella.*
(Fig. A-12. (1) conidiophore; (2) primary head with spores (conidia); (3) secondary head; (4) zygospore. Adapted from Blakeslee.)



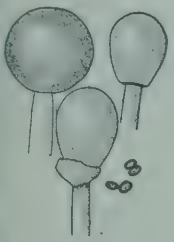
A-1
Rhizopus



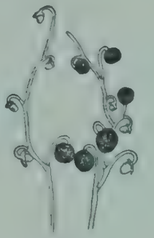
A-2
Absidia



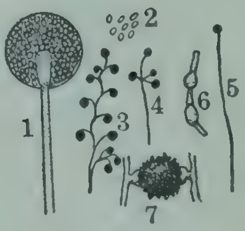
A-3
Actinomucor



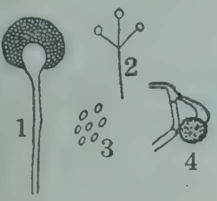
A-4
Phycomyces



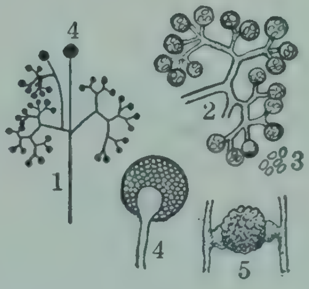
A-5
Circinella



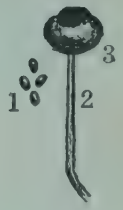
A-6
Mucor



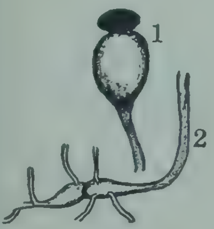
A-7
Zygorhynchus



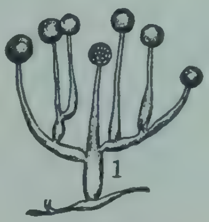
A-8
Thamnidium



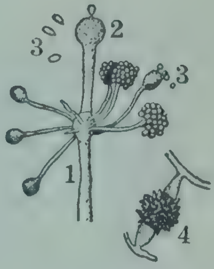
A-9
Pilaira



A-10
Pilobolus



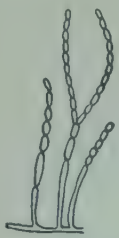
A-11
Mortierella



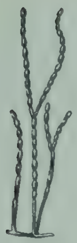
A-12
Cunninghamella

Key to Genera of Family Moniliaceae

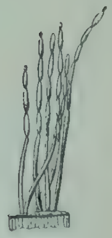
a. Conidia one-celled.
b. Conidiophores short or little differentiated from the conidia.
c. Conidiophores short, rarely branched.
d. Conidia not pointed, spherical or cylindric.
e. Conidia with rounded ends, conidiophores not erect. *Oospora*
(Fig. B-1. Conidiophores and conidia. After Saccardo)
2e. Conidia truncate, conidiophores erect. *Geotrichum*
(Fig. B-2. Conidiophores and conidia.)
2d. Conidia elongate with pointed ends. *Fusidium*
(Fig. B-3. Conidiophores and conidia.)
2c. Conidiophores longer and distinctly branched. *Monilia*
(Fig. B-4. Branched conidiophore and branching chains of conidia. Adapted from Went.)
2b. Conidiophores relatively long, well differentiated.
c. Conidiophores unbranched or only at tip, conidia in heads.
d. Conidia solitary, not in chains.
e. Conidia embedded in mucus or slime. *Hyalopus*
(Fig. B-5. (1) conidiophore with terminal globule of mucus in which conidia are enclosed; (2) conidiophores and conidia without mucus. Adapted from Nypels.)
2e. Conidia not embedded in mucus.
f. Conidiophores unbranched. *Cephalosporium*
(Fig. B-6. Mycelial thread with conidiophores and conidia. Adapted from Lindau.)
2f. Conidiophores branched, pointed. *Trichoderma*
(Fig. B-7. (1) branched conidiophores and conidial heads; (2) single head of conidia. Adapted from Harz.)
2d. Conidia in chains.
e. Conidiophore enlarged at tip, bearing sterigmata which in turn produce the spores. *Aspergillus*
(Fig. B-8. (1) conidiophore and head; (2) conidia; (3) detail of head.)
2e. Conidiophores not enlarged at tip.
f. Conidia not on sterigmata, barrel-shaped; conidiophores symmetrically branched. *Amblyosporium*
(Fig. B-9. Conidiophores and conidial chains. Adapted from Harz.)
2f. Conidia borne on sterigmata, spherical or elongate; conidiophores branched either symmetrically or asymmetrically.
g. Conidia not embedded in mucus.
h. Conidia without basal pore or ring. *Penicillium*
(Fig. B-10. Conidiophore with sterigmata and conidia.)
2h. Conidia with basal pore or ring. *Scopulariopsis*
(Fig. B-11.)
2g. Conidia embedded in globule of mucus, chains of spores not always easily distinguishable. *Gliocladium*
(Fig. B-12. (1) Conidiophore with sterigmata and conidia; (2) conidia each in globule of mucus; (3) large mucus globule resulting from fusion of smaller.)



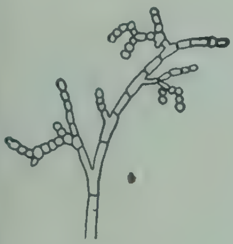
B-1
Oöspora



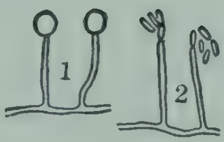
B-2
Geotrichum



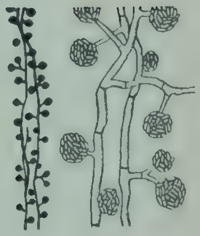
B-3
Fusidium



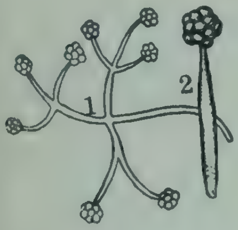
B-4
Monilia



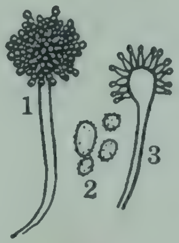
B-5
Hyalopus



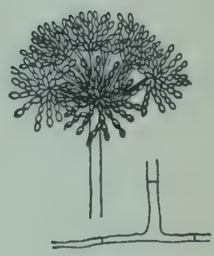
B-6
Cephalosporium



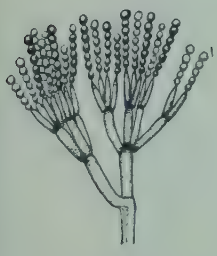
B-7
Trichoderma



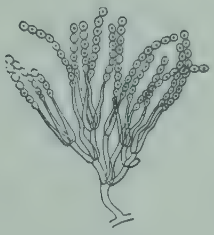
B-8
Aspergillus



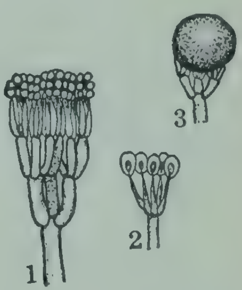
B-9
Amblyosporium



B-10
Penicillium

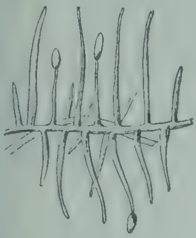


B-11
Scopulariopsis

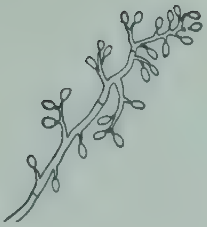


B-12
Gliocladium

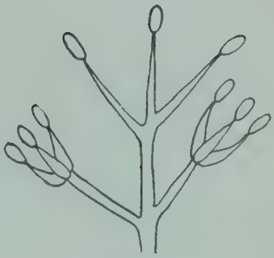
2c. Conidiophores either unbranched or not branched at tip only.
d. Conidia borne usually at tips of conidiophores, at least not on special intercalary cells.
e. Branches of conidiophores not in whorls.
f. Conidia smooth.
g. Conidiophores not erect.
h. Side branches of conidiophore erect, each with a single apical conidium. *Acremonium*
(Fig. C-1. Horizontal hyphae with conidiophores and conidia.)
2h. Side branches not erect, conidia both apical and lateral. *Sporotrichum*
(Fig. C-2. Conidiophore and conidia. Adapted from Harz.)
2g. Conidiophores erect.
h. Conidia globose or ovoid, not cylindric.
i. Conidia borne singly. *Monosporium*
(Fig. C-3. Conidiophore and conidia. Adapted from Saccardo.)
2i. Conidia in heads. *Botrytis*
(Fig. C-4. Conidiophores and conidia. Adapted from Frank.)
2h. Conidia cylindric. *Cylindrophora*
(Fig. C-5. Conidiophore and conidia.)
2f. Conidia not smooth, rough or warty. *Sepedonium*
(Fig. C-6. Conidiophores and conidia. Adapted from Tulasne.)
2e. Branches of conidiophores in whorls.
f. Apices of conidiophores sterile, conidia on short lateral branches. *Pachybasium*
(Fig. C-7. Conidiophore and conidia. Adapted from Oudemans.)
2f. All branches of conidiophore fertile.
g. Spores not in chains; solitary or in heads.
h. Conidia spherical or short.
i. Conidia not embedded in mucus.
j. Branches of conidiophores not in pairs at right angles to each other. *Verticillium*
(Fig. C-8. Conidiphores and conidia. Adapted from Harz.)
2j. Branches of conidiophores at right angles. *Verticilliastrum*
(Fig. C-9.)
2i. Conidia embedded in mucus. *Acrostalagmus*
(Fig. C-10. (1) conidiophore and conidia; (2) tip of conidiophore; (3) attachment of conidia. Adapted from Corda.)
2h. Spores elongate, cylindric or long spindle-shaped. *Acrocylindrium*
(Fig. C-11. Conidiophores and conidia.)
2g. Conidia in chains, on sterigmata. *Spicaria*
(Fig. C-12. (1) sterigmata with conidia; (2) conidiophore with conidia. Adapted from Oudemans.)



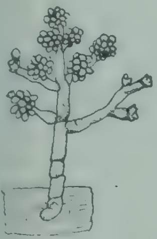
C-1
Acremonium



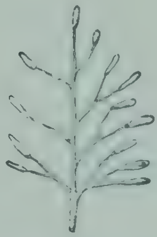
C-2
Sporotrichum



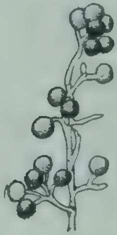
C-3
Monosporium



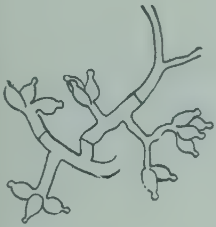
C-4
Botrytis



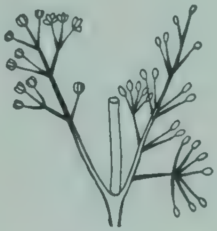
C-5
Cylindrophora



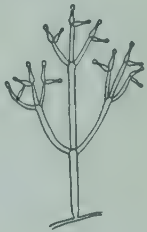
C-6
Sepedonium



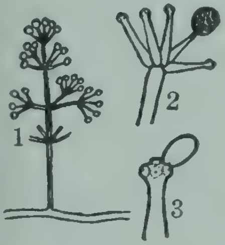
C-7
Pachybasium



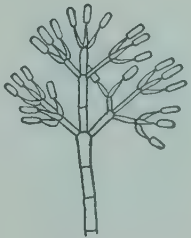
C-8
Verticillium



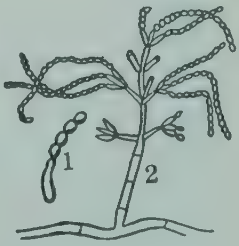
C-9
Verticilliastrum



C-10
Acrostalagmus



C-11
Acrocylindrium



C-12
Spicaria

2d. Conidiophores with special intercalary cells on which conidia are borne *Nematogonum*

(Fig. D-1. Conidiophores and conidia. Adapted from Saccardo.)

2a. Conidia two or more celled.

b. Conidia two-celled.

c. Conidia on short lateral conidiophores, solitary, rough. *Mycogone*

(Fig. D-2. Conidiophores and conidia. Adapted from Saccardo.)

2c. Conidia terminal, frequently clustered. *Trichothecium*

(Fig. D-3. Conidiophores and conidia. Adapted from Matruchot.)

2b. Conidia more than two-celled, conidia elongate, conidiophores branched in whorls. *Dactylium*

(Fig. D-4. Conidiophores and septate conidia.

Key to the Genera of the Family Dematiaceae

a. Conidia one-celled.

b. Both conidia and conidiophores dark-colored.

c. Conidia not in chains.

d. Conidia in terminal heads.

e. Conidia at end of conidiophores.

f. Conidia not embedded in mucus. *Stachybotrys*

(Fig. D-5. Conidiophores and conidia. Adapted from Oudemans.)

2f. Conidia embedded in mucus. *Gliobotrys*

(Fig. D-6. Conidiophores, with embedded conidia.)

2e. Conidia irregularly distributed at and near the tip.

f. Conidiophores somewhat inflated at tip, conidia spherical. *Periconia*

(Fig. D-7. (1) conidiophore with conidial head; (2) detail of spore attachment. Adapted from Saccardo.)

2f. Conidiophores not inflated, conidia elongate. *Synsporium*

(Fig. D-8. Conidiophore and conidia. Adapted from Cavara.)

2d. Conidia not in terminal heads.

e. Conidia sessile both sides and tip conidiophore. *Trichosporium*

(Fig. D-9. Branched conidiophore and conidia.)

2e. Conidiophores branching, conidia solitary.

f. Conidia on short lateral branches of mycelium.

g. Conidiophores not inflated. *Acremoniella*

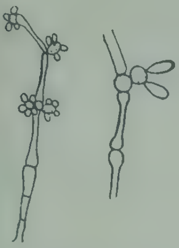
(Fig. D-10. Mycelial thread with lateral conidiophores and conidia. Adapted from Marchal.)

2g. Conidiophores inflated at tip. *Nigrospora*

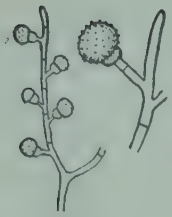
(Fig. D-11.)

2f. Conidia on well-branched conidiophores. *Monotospora*

(Fig. D-12.)



D-1
Nematogonum



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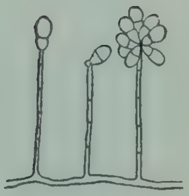
Mycogone



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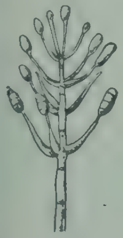
Trichothecium



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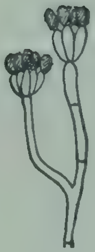
Dactylium



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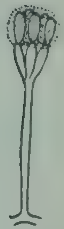
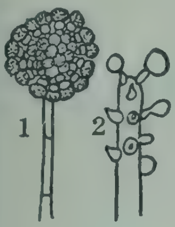
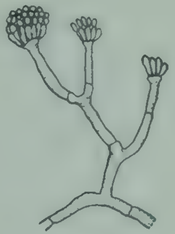
Stachybotrys



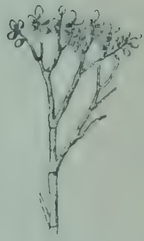
D-6
Gliobotrys



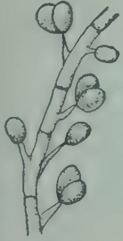
D-7
Periconia



D-8
Synsporium



D-9
Trichosporium



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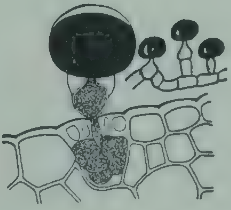
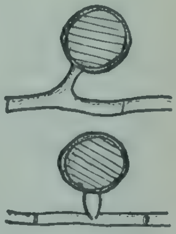
Acremoniella

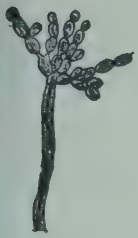


D-11
Nigrospora



D-12
Monotospora

2c. Conidia in chains.
d. Conidial chains branched. *Hormodendrum*
(Fig. E-1. Conidiophores and conidia. Adapted from Brahne.)
2d. Conidia in unbranched chains from tips of whorled apical sterigmata. *Haplographium*
(Fig. E-2. Conidiophores and conidia.)
2b. Conidiophores dark, conidia clear or bright-colored.
c. Conidia in heads. *Stachylidium*
(Fig. E-3. Conidiophore and globular conidia.)
2c. Conidia terminal, not in heads.
d. Conidia globose, conidiophores at base of sterile hypha. *Botryotrichum*
(Fig. E-4. Conidiophores with sterile tips and basal conidia.)
2d. Conidia ovate; on lower lateral branches of conidiophores with upper branches sterile. *Mesobotrys*
(Fig. E-5. Conidiophores and conidia. Adapted from Abbott.)
2a. Conidia more than one-celled, dark in color.
b. Conidia two-celled.
c. Conidiophores very short, little differentiated. *Dicoccum*
(Fig. E-6. Conidiophore and conidia. Adapted from Harz.)
2c. Conidiophores distinct, usually erect.
2d. Conidia in chains.
e. Long chains of conidia. *Diplococcium*
(Fig. E-7. Conidiophores and long branched chains of conidia.)
2e. Chains of conidia short. *Cladosporium*
(Fig. E-8. Conidiophores and conidia. Adapted from Janczewski.)
2d. Conidia single, borne on short terminal phialides on short conidiophores. *Scolecobasidium*
(Fig. E-9. Conidiophores, hairlike phialides, conidia. Adapted from Abbott.)
2b. Conidia more than two-celled.
c. Conidia with transverse septa only.
d. Conidiophores well differentiated.
e. Conidia not in whorls.
f. Conidia with more than five cells. *Helminthosporium*
(Fig. E-10. Conidiophores and conidia. Adapted from Saccardo.)
2f. Conidia 4 or 5 celled. *Curvularia*
(Fig. E-11.)
2e. Conidia in whorls.
f. Whorls lateral. *Spondylocladium*
(Fig. E-12. Conidiophore and conidia. Adapted from Preuss.)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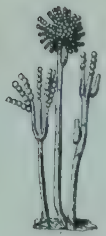
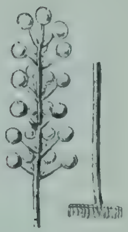
Hormodendrum


E-2
Haplographium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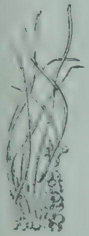
Stachylidium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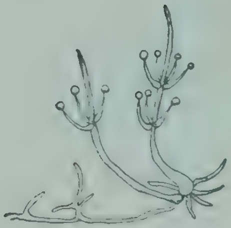
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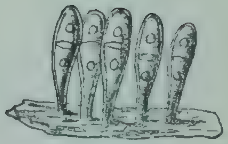
Mesobotrys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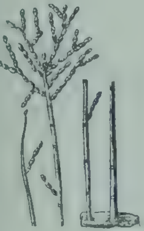
Dicoccum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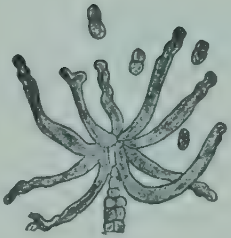
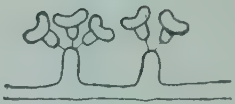
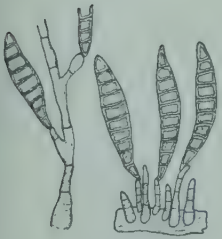
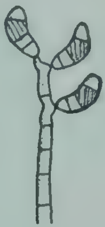
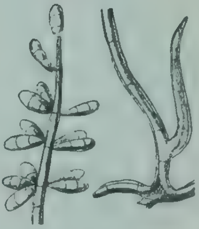
Diplococcium


E-8
Cladosporium


E-9
Scolecobasidium


E-10
Helminthosporium


E-11
Curvularia


E-12
Spondylocladium

2f. Whorls apical only. *Acrothecium*

(Fig. F-1. Conidiophore and conidia. Adapted from Saccardo.)

2d. Conidiophores very short or absent. *Clasterosporium*

(Fig. F-2. Conidiophore and conidia of different ages. Adapted from Saccardo.)

2c. Conidia with longitudinal septa, or with both longitudinal and transverse.

d. Conidia not in chains.

e. Conidia wtih longitudinal septa only, 4 celled. *Tetracoccosporium*

(Fig. F-3. Conidiophores and conidia. Adapted from von Szabo.)

2e. Conidia with both transverse and longitudinal septa.

f. Conidiophores not erect. *Stemphylium*

(Fig. F-4. Conidiophore and conidia. Adapted from Saccardo.)

2f. Conidiophores erect.

g. Conidia solitary, large. *Macrosporium*

(Fig. F-5. Conidiophores and conidia. Adapted from Berlese.)

2g. Conidia several at tip of conidiophore. *Dactylosporium*

(Fig. F-6. Conidiophores and conidia. Adapted from Corda.)

2d. Conidia in chains; with both transverse and longitudinal walls. *Alternaria*

(Fig. F-7. Conidiophores and conidia. Adapted from Saccardo.)

Key to Genera of the Family Stilbaceae

a. Hyphae, coremium and conidia hyaline or bright-colored.

b. Conidia not in chains.

c. Conidia in head or heads.

d. Coremium with heads at tip. *Stilbella*

(Fig. F-8. Coremium.)

2d. With both terminal and lateral heads on coremium. *Tilachlidium*

(Fig. F-9. (1) coremium and separate conidiophores and conidial heads; (2) conidial heads; (3) conidia. Adapted from Oudemans.)

2c. Conidia not in heads.

d. Conidia terminal. *Ciliciopodium*

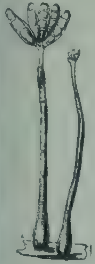
(Fig. F-10. Coremium and separate conidiophores.)

2d. Conidia both terminal and lateral. *Isaria*

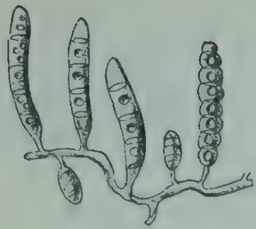
(Fig. F-11. (1) coremium; (2) conidiophore. Adapted from Saccardo.)

2b. Conidia in chains. *Coremium*

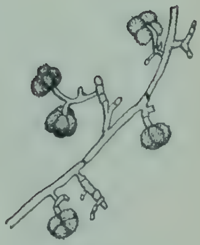
(Fig. F-12. Coremium and single conidiophore.)



F-1
Acrothecium



F-2
Clasterosporium



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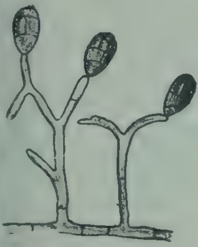
Tetracoccosporium



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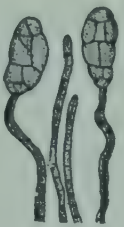
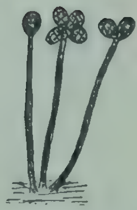
Stemphylium



F-5
Macrosporium



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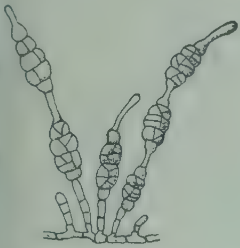
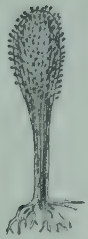
Dactylosporium



F-7
Alternaria



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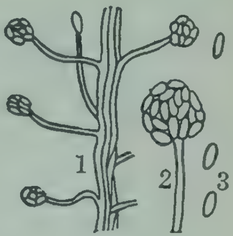
Stilbella



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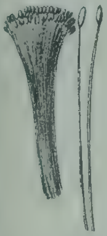
Tilachlidium



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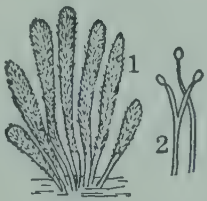
Ciliciopodium



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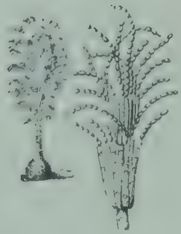
Isaria

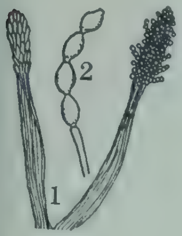


F-12
Coremium

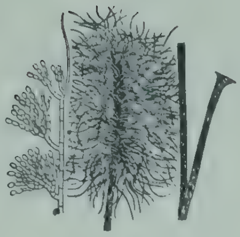
2a. Hyphae, coremia and conidia dart.
  b. Conidia not embedded in mucus.
    c. Chains of conidia not mixed with sterile hairs. *Stysanu*
      (Fig. G-1. (1) coremium; (2) conidiophore with chain of conidia. Adapted from Lindau.)
    2c. Chains of conidia mixed with sterile hairs or setae. *Trichurus*
      (Fig. G-2.)
  2b. Conidia embedded in mucus. *Metarrhizium*
    (Fig. G-3.)

Key to Genera of Family Tuberculariaceae

a. Conidia and hyphae hyaline or light-colored.
  b. Conidia one-celled.
    c. Sporodochium without spines. *Hymenula*
      (Fig. G-4. (1) Conidiophores; (2) conidia; (3) sporodochia. Adapted from Boudier.)
    2c. Sporodochium with marginal spines or hairs. *Volutella*
      (Fig. G-5. (1) conidiophores and conidia; (2) sporodochium with spines. Adapted from Boulanger.)
  2b. Conidia several-celled, sickle or crescent-shaped. *Fusarium*
    (Fig. G-6. (1) conidiophores and conidia; (2) sporodochium. Adapted from Saccardo.)
2a. Conidia or hyphae dark or smoky.
  b. Conidia one-celled.
    c. Sporodochium surrounded by hyaline hairs or bristles. *Myrothecium*
      (Fig. G-7.)
    2c. Sporodochium punctate, gelatinous. *Epicoccum*
      (Fig. G-8. Sporodochium and conidia. Adapted from Penzig.)
  2b. Conidia several-celled. *Thyrococcum*
    (Fig. G-9. Sporodochium and conidia.)



G-1
Stysanus



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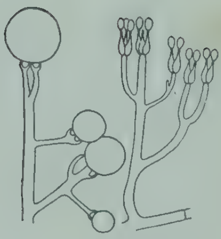
Trichurus



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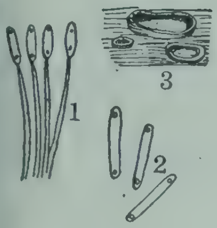
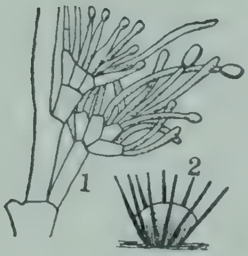
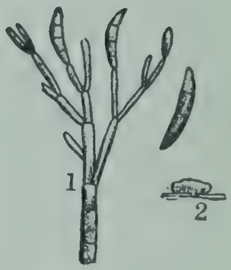
Metarrhizium



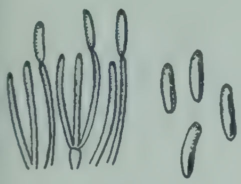
G-4
Hymenula



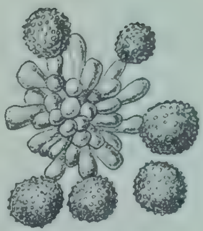
G-5
Volutella



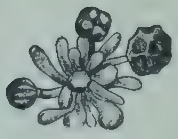
G-6
Fusarium



G-7
Myrothecium



G-8
Epicoccum



G-9
Thyrococcum

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